REMARKS/ARGUMENTS

By the present amendment, claim 1 has been amended and previous claims 4 and 5 have been deleted rendering claims 1, 6-10 and 12-13 pending in the application. The amendments to the claims have been made without prejudice and without acquiescing to any of the Examiner's objections. Applicant reserves the right to pursue any of the deleted subject matter in a further continuation, continuation-in-part or divisional application. The amendment does not contain new matter and its entry is respectfully requested.

The Official Action dated November 26, 2003 has been carefully considered. It is believed that the amended claims submitted herewith and the following comments represent a complete response to the Examiner's rejections and place the present application in condition for allowance. Reconsideration is respectfully requested.

Finality of Office Action

The Examiner has made this office action final which is improper as the Examiner has raised new grounds of rejection. In particular, the Examiner has now raised new objections under 35 USC §112, first paragraph under both written description and enablement. Applicants' last amendment did not necessitate the new ground of rejection as in the last amendment the claims were amended in order to introduce additional features from the dependent claims. Therefore, we respectfully request that the finality of the office action be withdrawn as being premature.

35 USC §112, First Paragraph

The Examiner has objected to claims 1, 4-10 and 12-13 under 35 USC §112, first paragraph as failing to comply with the written description requirement.

The Examiner acknowledges that the "specification as filed discloses a reporter gene construct comprising an enhancer element (Sox9 binding sequence upstream of the mouse Col II minimal promoter, -89 to +13) that is responsive to the transcription factor

Sox9 (spec. page 17, Example 1)". However, the Examiner alleges that "the specification as filed fails to disclose any other genetic construct that comprises any other nucleotide acid sequence that binds to any other endogenous protein in the cells". In order to expedite prosecution, claim 1 has been amended, without prejudice, in order to specify that the reporter gene comprises a sequence upstream of the promoter from the type II collagen gene which the Examiner admits meets the written description requirements.

We disagree with the Examiner's statement that the "specification as filed fails to disclose what comprises the claimed pGL3(4X48) nucleic acid construct, and fails to identify any 48-bp fragment (Sox9 response element) that is responsive to transcription factor Sox9". The preparation of the reporter plasmids is clearly described in Example 1 of the application as published on page 6, paragraph 0088. In particular, this section discloses that the Sox9 responsive reporter gene from the 4X48-p89 luciferase was subcloned into pGL3-basic. The 4X48-p89 luciferase is explicitly described in reference 14 as mentioned on page 6, line 2 of paragraph 0088 of the application as published. We point out that all of the references, including reference 14, are incorporated by reference in their entirety as mentioned on page 8, paragraph 0108 of the published application. Reference 14 is a LeFebvre et al. publication and we enclose a copy for the Examiner's benefit although it was provided with the Information Disclosure Statement. Referring to Figure 1 of this article, the Examiner will note that the 4X48-luciferase construct is completely described. In particular, the nucleic acid sequence of the 48-bp fragment is provided both in Figures C and D. The inventors took this 4X48-p89 luciferase construct and sub-cloned it into pGL3-basic as described on page 6, lines 3-9 of paragraph 0088 of the published application. The pGL-3 basic is readily available from Promega. To generate the EGFP-based reporter, the luciferase gene within pGL3(4X48) was replaced with EGFP-N1 which is readily available from Clontech. Therefore, one of ordinary skill in the art would be readily able to construct the reporter constructs described in the present application, without undue experimentation. A deposit of the constructs is not required in the circumstances.

In view of the foregoing, we respectfully request that the objections to the claims under 35 USC §112, first paragraph as lacking written description, be withdrawn.

The Examiner has also objected to claims 1, 4, 9-10 and 12-13 under 35 USC §112, first paragraph as not providing reasonable "enablement for the method (as claimed) that requires the use of any response element which binds to any endogenous protein in mesenchymal cells in the process of chondrogenesis".

The Examiner does agree that the specification is "enabling for method of identifying a modulator of chondrogenesis by transiently transfecting the primary limb mesenchymal cells with a nucleic acid construct comprising Sox9 response element *(Col2a1)* operatively linked to a reporter gene". Consequently, in order to expedite prosecution, the claims have been amended, without prejudice, in order to specify that the nucleic acid construct comprises the Sox9 response element from the type II collagen gene.

The Examiner also comments on page 7 of the office action that the specification as filed fails to disclose what comprises the claimed pGL3(4X48) nucleic acid construct, since it fails to disclose what are the structural and/or functional limitations of pGL3(4X48). As mentioned above under the written description objection, the present application does provide clear and sufficient guidance to enable one of skill in the art to prepare the claimed constructs.

In view of the foregoing, we respectfully request that the objections to the claims under 35 USC §112, first paragraph as lacking enablement, be withdrawn.

35 USC §103

The Examiner has objected to claims 1, 4-8 and 12-13 under 35 USC §103 as being unpatentable over LeFebvre et al. (Matrix Biology 16:529-540, 1998) or LaFebvre et al.

(EMBO J. 17(19):5718-5733, 1998) in view of Healy et al. (Dev. Dyn. 215:68-78, 1999). We respectfully disagree with the Examiner for the reasons that follow.

The LeFebvre references disclose that the transcription factor Sox9 is expressed during chondrogenesis and activates the chondrocyte specific *Col2a1* enhancer. In LeFebvre's experiments, Sox9 is transfected in the cells. LeFebvre does not describe a screening assay wherein a test compound is added to cells transfected with a reporter construct containing *Col2a1* and wherein endogenous Sox9 activity is assessed. Healey et al. describe the role of Sox9 in limb development. Healey et al. transfected Sox9 into developing chick limbs using retroviral transfection. Healey et al. does not describe a screening assay wherein a test compound is added to cells transfected with a reporter construct containing *Col2a1* and wherein endogenous Sox9 activity is assessed.

As the Examiner is aware, he has the initial burden of factually supporting any *prima facie* conclusion of obviousness. To establish a *prima facie* case of obviousness, three basic criteria must be met. First, the prior art references when combined must teach or suggest all the claim limitations. Second, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Finally, there must be a reasonable expectation of success. We respectfully submit that the above criteria have not been met as explained below.

1) Prior art does not teach all claim limitations

The method of the invention relates to a **method of identifying a modulator** of chondrogenesis. As required by claim 1, a test compound is added to the assay to determine its effect on chondrogenesis. None of the cited art relates to screening assays for identyfing modulators of chondrogenesis. Therefore, this limitation is not taught in the prior art.

The method of the invention uses **transient transfection of primary limb mesenchymal cells** in the screening method. None of the cited art describes screening assays for identifying modulators of chondrogenesis using primary limb mesenchymal cells. Therefore, this limitation is not taught in the prior art.

The method of the invention measures **expression of endogenous Sox9**. The cited art involves transfecting the cells with Sox9. Therefore, this limitation is not taught in the prior art.

2) Suggestion or motivation in the prior art

None of the references cited by the Examiner suggest or provide motivation to develop a method for identifying a modulator of chondrogenesis as claimed in the present application. In fact, since the combination of the references does not teach all of the features of the claims even if the references were combined, the claimed invention would not be achieved. In addition, as discussed below, one of skill in the art would not be motivated to modify the prior art in order to achieve the present invention as there was no reasonable expectation of success at the time of the invention.

3) No reasonable expectation of success

As mentioned previously, the screening assay of the invention is built on several novel and unobvious features which were not taught in the prior art. These features include 1) the transient transfection method which involves transfection of the primary limb mesenchymal cells in suspension at the time of seeding; 2) the use of primary limb mesenchymal cells and transient transfection to assess the effect of heterologous gene expression on chondrogenesis; and 3) the use of a Sox-9 reporter to follow chondrogenesis in a cell autonomous manner. With respect to this final point, no one had shown at the time of the invention that factors (other than SOX9) that stimulate chondrogenesis would stimulate a Sox9-responsive reporter gene in mesenchymal cells. This is an important consideration for the following reasons.

Primary limb mesenchymal cells have the potential to contribute to multiple mesoderm-derived lineages (i.e. bone, cartilage, fat, muscle, etc.) and within these cultures numerous cells types form in addition to chondrocytes. Chondrogenesis begins with the aggregation of prechondrogenic cells into precartilaginous aggregations, cells within these aggregates subsequently differentiate into chondroblasts which give rise to the cartilage nodules present in the culture. As indicated, in amongst these nodules other cell types are apparent, fibroblasts, adipoblasts and myoblasts, etc. In order to study the effect of various genes on chondrogenesis, retroviruses harboring the gene of interest would be used to infect these cultures. In this manner, most of the cells would be infected and expressing the gene of interest and the consequences of the expression of this gene could be assessed visually or by staining with alcian blue (a cartilage matrix stain). It was also assumed that to examine the effect of a gene-product on chondrogenesis it would be necessary to express the genes in most of the cells (using a retroviral-based approach) to insure that most of the cells within a condensation expressed the gene. With transient transfection, at best, only 1-5% of the cells express the gene of interest and for genes that act intracellularly (i.e. transcription factors, signaling pathways, receptors) it was assumed no effect would be observed as insufficient prechondrogenic cells would be expressing the gene of interest. To circumvent this problem, the inventors transfected the cells with a Sox9-responsive reporter gene and, in a specific embodiment, coupled the transfection with a gene of interest. In this manner, the inventors can follow what is happening to chondrogenesis in the small numbers of transfected cells that are scattered throughout the culture. It was surprising and unexpected to the inventors that this approach actually worked. It was initially assumed this strategy wouldn't work, because of the well-demonstrated need in previous studies to obtain a large percentage of expressing cells to obtain a measurable effect on chondrogenesis. In this regard, we enclose a copy of Hall and Miyake, BioEssays 22:138-147, 2000, with relevant sections boxed. Hall and Miyake affirm that chondroblast differentiation only occurs in condensations of a suitable size in culture. In this regard, we refer to page 144, lines 1-3 of paragraph 2 and page 143, lines 1-6 of paragraph 3. Furthermore, the transfection procedure of the invention

allows prechondrogenic cells (in the centre of the culture) to be transfected, whereas traditional transfection approaches led to transfection of only cells within the periphery of the culture. Thus, at the time of the invention, there was no reasonable expectation of success for the claimed screening assay based on what is known about the nature of the primary limb mesenchymal cells and the process of chondrogenesis.

In addition to the above three criteria, the Examiner is also required to consider secondary considerations, such as unexpected results and long-felt need when assessing obviousness. In this regard, we submit that the method of the present invention does provide a simple and efficient screening assay in order to identify modulators of chondrogenesis. Traditionally, retroviral infection has been used to test the ability of various gene products to stimulate chondrogenesis in primary limb mesenchymal cells. This method is laborious, cumbersome and not conducive to high throughput as the generation of retroviral particles is a time-consuming process. In this regard, we point out that Healy et al. (which is cited by the Examiner) teach the use of retroviral infection of mesenchymal cells to examine the effect of Sox9 on chondrogenesis. This approach relies on retroviruses and does not teach the use of transient transfection coupled with a Sox9-responsive reporter gene to examine chondrogenesis. Retroviral infection and transient transfection rely on distinct approaches for heterologous gene expression. Furthermore, retroviruses are not typically combined with reporter genes. The use of transient transfection coupled with a reporter gene affords the use of co-transfection of expression plasmid(s) containing gene(s) of interest along with a reporter gene. The transient transfection method described in the application was not taught in the prior art and is a unique process developed by the applicants. This simple and efficient reporter-gene based assay provides a means in which to measure the consequences of gene expression on chondrogenesis.

In view of the foregoing, we respectfully submit that the screening method as claimed in the present application is not obvious in view of any of the cited references, either alone

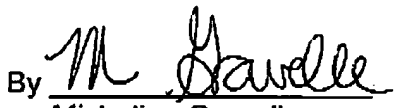
or in combination and we respectfully request that the objection to the claims under 35 USC §103 be withdrawn.

The Commissioner is hereby authorized to charge any fee (including any claim fee) which may be required to our Deposit Account No. 02-2095.

In view of the foregoing comments and amendments, we respectfully submit that the application is in order for allowance and early indication of that effect is respectfully requested. Should the Examiner deem it beneficial to discuss the application in greater detail, he is kindly requested to contact the undersigned by telephone at (416) 957-1682 at his convenience.

Respectfully submitted,

BERESKIN & PARR

By 
Micheline Gravelle
Reg. No. 40,261

Bereskin & Parr
Box 401, 40 King Street West
Toronto, Ontario
Canada M5H 3Y2
Tel: 416-957-1682
Fax: 416-361-1398

Attachments

MOLECULAR AND CELLULAR BIOLOGY, Aug. 1996, p. 4512–4523
0270-7306/96/$04.00+0

Vol. 16, No. 8

An 18-Base-Pair Sequence in the Mouse Proα1(II) Collagen Gene Is Sufficient for Expression in Cartilage and Binds Nuclear Proteins That Are Selectively Expressed in Chondrocytes

VÉRONIQUE LEFEBVRE, GUANG ZHOU, KRISH MUKHOPADHYAY, CHAD N. SMITH, ZHAOPING ZHANG, HEIDI EBERSPAECHER, XIN ZHOU, SATRAJIT SINHA, SANKAR N. MAITY, AND BENOIT DE CROMBRUGGHE*

Department of Molecular Genetics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Received 19 March 1996/Returned for modification 29 April 1996/Accepted 8 May 1996

The molecular mechanisms by which mesenchymal cells differentiate into chondrocytes are still poorly understood. We have used the gene for a chondrocyte marker, the proα1(II) collagen gene (*Col2a1*), as a model to delineate a minimal sequence needed for chondrocyte expression and identify chondrocyte-specific proteins binding to this sequence. We previously localized a cartilage-specific enhancer to 156 bp of the mouse *Col2a1* intron 1. We show here that four copies of a 48-bp subsegment strongly increased promoter activity in transiently transfected rat chondrosarcoma (RCS) cells and mouse primary chondrocytes but not in 10T1/2 fibroblasts. They also directed cartilage specificity in transgenic mouse embryos. These 48 bp include two 11-bp inverted repeats with only one mismatch. Tandem copies of an 18-bp element containing the 3′ repeat strongly enhanced promoter activity in RCS cells and chondrocytes but not in fibroblasts. Transgenic mice harboring 12 copies of this 18-mer expressed luciferase in ribs and vertebrae and in isolated chondrocytes but not in noncartilaginous tissues except skin and brain. In gel retardation assays, an RCS cell-specific protein and another closely related protein expressed only in RCS cells and primary chondrocytes bound to a 10-bp sequence within the 18-mer. Mutations in these 10 bp abolished activity of the multimerized 18-bp enhancer, and deletion of these 10 bp abolished enhancer activity of 465- and 231-bp intron 1 segments. This sequence contains a low-affinity binding site for POU domain proteins, and competition experiments with a high-affinity POU domain binding site strongly suggested that the chondrocyte proteins belong to this family. Together, our results indicate that an 18-bp sequence in *Col2a1* intron 1 controls chondrocyte expression and suggest that RCS cells and chondrocytes contain specific POU domain proteins involved in enhancer activity.

Acquisition of the chondrocyte phenotype by mesenchymal cells is one of the major pathways of differentiation of these cells. Chondrocytes form several types of cartilages including the growth plate cartilages essential to skeletal formation and cartilages that have supporting roles and persist throughout adult life such as the articular cartilages and the cartilages of the nose, ear, and trachea. Chondrocyte differentiation presumably involves first the commitment of undifferentiated mesenchymal cells to the chondrocyte lineage (1). Cell condensation and further maturation lead to a fully differentiated phenotype characterized by the synthesis of cartilage extracellular matrix proteins, including collagen types II, IX, and XI, the large proteoglycan aggrecan, the link protein, and the cartilage oligomeric protein (24). Recent molecular and biochemical studies with cell culture, gene inactivation experiments with mice, and the identification of genes responsible for mouse and human skeletal abnormalities have documented the importance of growth and differentiation factors, extracellular matrix proteins, signaling mediators, and transcription factors in skeletal development (5, 23). However, no specific transcription factors that control the differentiation of chondrocytes from mesenchymal cells and activate chondrocyte-specific genes have yet been identified.

Type II collagen is the most abundant extracellular protein made by chondrocytes. Its essential structural role in cartilage is best illustrated by the severe skeletal anomalies shown by humans and mice carrying mutant proα1(II) collagen chains (7, 25). The type II collagen gene starts to be expressed following mesenchymal cell condensation that precedes cartilage formation, and thus it represents an early marker of chondrocyte differentiation. The type II collagen gene is also expressed transiently in some extrachondrogenic sites during embryonic development, including the notochord, heart, epidermis, and discrete areas of the brain (2). However, expression at these sites is low, and the role of type II collagen in these extrachondrocytic sites is not understood. The type II collagen gene should therefore be an excellent model for studies of chondrocyte-specific transcriptional mechanisms.

Previous DNA transfection studies showed that 620 bp of the first intron of the rat Col2a1 gene enhanced promoter activity specifically in primary chick chondrocytes (11). Later studies of this enhancer pinpointed a 260-bp sequence that enhanced promoter activity sixfold in chondrocytes (26). In another study, two silencer elements were located in the rat *Col2a1* promoter and were proposed to inactivate the gene in nonchondrocytic cells (21). Experiments with transgenic mice indicated that the first intron of the rat Col2a1 gene was necessary to direct cartilage-specific activity of a 3-kb *Col2a1* promoter (27). In another study, the DNA extending from 3 kb upstream of the start of transcription to exon 4 of the mouse Col2a1 gene conferred a pattern of *lacZ* expression in transgenic mouse embryos that coincided with the chondrocytic

* Corresponding author. Mailing address: Department of Molecular Genetics, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Phone: (713) 792-2590. Fax: (713) 794-4295.

expression of the endogenous gene during embryonic development, and deletion of intron 1 inhibited expression of the transgene (15).

Our laboratory has recently started to reexamine the *cis*-acting elements that direct expression of the mouse Col2a1 gene in chondrocytes. In one approach we generated and studied transgenic mice (28), and in another, parallel approach we performed transient expression experiments in rat chondrosarcoma (RCS) cells, mouse primary chondrocytes, and, as controls for nonchondrogenic cells, 10T1/2 fibroblasts and C_2C_{12} myoblasts (17). RCS cells are a stable and fully differentiated chondrocyte cell line that synthesizes type II, IX, and XI collagens as well as cartilage-specific proteoglycans (17). Unlike other so-called chondrocyte cell lines, they contain no type I collagen RNA. They also contain no type X collagen RNA, suggesting that they were frozen in a stage of chondrocyte differentiation that precedes hypertrophy. Analysis of progressively shorter *Col2a1* intron 1 segments revealed that two tandem copies of a 182-bp fragment were sufficient for cartilage expression in transgenic mice and that two tandem copies of a 156-bp fragment, included in the 182 bp, were able to strongly increase promoter activity in RCS cells but not in 10T1/2 fibroblasts. Further deletions suggested that the 3′ and 5′ parts of the 156- and 182-bp segments might be necessary for chondrocyte expression. We also determined that the Col2a1 promoter was dispensable for chondrocyte expression (17, 28). Indeed, strong promoter activation was still obtained in chondrocytes in both transgenic mice and transient transfections when a 182-bp enhancer or a 231-bp intron fragment containing the 182 bp was cloned upstream of a minimal heterologous promoter, either a β-globin promoter or the adenovirus major late promoter.

In the present study, we aimed at delineating more precisely the *cis*-acting elements in *Col2a1* intron 1 that are needed for chondrocyte expression and also asked whether chondrocyte-specific proteins were binding to these elements. We show that multiple copies of an 18-bp subsegment of the 156-bp enhancer can strongly enhance promoter activity selectively in transiently transfected RCS cells and chondrocytes and are also sufficient to direct promoter activity in chondrocytes of transgenic mice. Evidence that nuclear proteins present selectively in primary chondrocytes and RCS cells bind within this 18-mer to a 10-bp sequence which is essential for enhancer activity and that these proteins likely belong to the POU domain protein family is presented.

MATERIALS AND METHODS

Cell cultures. RCS cells were given by J. H. Kimura (Henry Ford Hospital, Detroit, Mich.), and ROS 17/2.8 cells were given W. T. Butler (The University of Texas Health Science Center, Houston). Rib chondrocytes were isolated from newborn mice as previously described (14). Other cell lines were from the American Type Culture Collection (Rockville, Md.). Cells were cultured under standard conditions (17).

Transient transfections. DNA transfections were carried out as described previously (17). Luciferase reporter plasmids were cotransfected with the pSV2βgal plasmid used as an internal control for transfection efficiency. Luciferase and β-galactosidase activities were assayed as described elsewhere (17). Differences between several experiments in the values obtained for a given construction transfected in a given cell type can be explained by assay variations from one experiment to another. Luciferase activities were expressed as 2×10^4 luciferase units per β-galactosidase unit. Luciferase values in extracts of RCS cells transfected with the 89-bp *Col2a1* promoter alone were never more than twice the blank; values with active enhancer elements were between 50- and 20,000-fold higher depending on the constructions used.

Col2a1-luciferase constructions. All final Col2a1 constructions were cloned in the pLuc4 vector (18). For intermediate construction steps, *Col2a1* intron 1 segments were cloned in the p89Col2a1Bs plasmid. This vector was obtained by cloning the 89-bp *Col2a1* promoter (−89 to +6) containing blunt-ended *Hin*dIII sites at both ends (17) between the *Eco*RV site and the blunt-ended *Xho*I site of pBluescript II Ks (+/−) (Stratagene, La Jolla, Calif.). Ligation of blunt-ended *Hin*dIII and *Xho*I sites reconstituted a *Hin*dIII site 3′ of the promoter. Single or tandem copies of intron 1 segments were cloned together with the 89-bp *Col2a1* promoter upstream of the luciferase gene in pLuc4.

Single copies or duplicate tandem copies of *Col2a1* intron 1 segments of 156, 231, and 465 bp were obtained as previously described (17). Other intron 1 segments were synthesized as double-stranded oligonucleotides containing a *Bam*HI site at the 5′ end and a *Bgl*II site followed by an *Eco*RI site at the 3′ end. These oligonucleotides were cloned between the *Bam*HI and *Eco*RI sites of p89Col2a1Bs. Dimers were obtained by cloning a second oligonucleotide molecule in the *Bgl*II and *Eco*RI sites. Tetramers were obtained by releasing the two oligonucleotide copies by *Bam*HI and *Eco*RI digestion and inserting them in a vector containing two copies and cut at the *Bgl*II and *Eco*RI sites. Further multimerization was done as for tetramers.

Plasmid p41Col1a2, originally called pI139 (8), contained a minimal *Col2a1* promoter cloned between the *Asp*718 and *Hin*dIII sites of pA$_3$LUC. Twelve copies of the R2 segment of *Col2a1* were introduced in this vector between the *Spe*I and *Bam*HI sites.

A 10-bp deletion within *Col2a1* intron 1 fragments was obtained by PCR. All constructions made with oligonucleotides and products of PCR were verified by DNA sequencing.

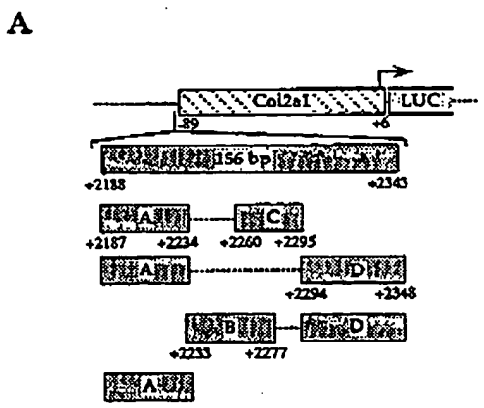
Transgenic mice. Two DNA constructions were made by cloning a four-copy A element or a 12-copy R2 element as a blunt-ended *Bam*HI-*Bgl*II fragment in the blunt-ended *Spe*I site of p309Col2a1 (28). This vector contained a 309-bp *Col2a1* promoter and the SA-βgeo-bpA cassette (6). For a third construction, the 12-copy R2 element was cloned upstream of an 89-bp *Col2a1* promoter in the pLuc4 vector. DNAs were released by restriction enzyme digestion 5′ of *Col2a1* sequences and 3′ of the polyadenylation signal located downstream of the reporter genes. Transgenic mice harboring these DNAs were generated as described elsewhere (28). Transgenic founder mice were sacrificed at day 14.5 of embryonic development or within 3 days after birth. Southern analysis, staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), measurements of luciferase activity in tissue extracts, and histology studies were performed as previously described (18, 28).

Nuclear extracts. Nuclear extracts from mouse chondrocytes were prepared as described elsewhere (4), either directly after isolation of the cells from cartilage or after up to 3 days in primary culture. One day before harvest of primary cells, ascorbic acid was added to culture media (17). Nuclear extracts from all other cell types were prepared as previously described (3) with 10 μg of leupeptin and pepstatin per ml in all buffers.

Gel retardation assays. The wild-type and mutant R2 probes were made by annealing complementary oligonucleotides as described in the legend to Fig. 5A. The OCT probe was made with oligonucleotides (5′-ggCCTGGGTAATTTGC ATTTCTAAAA-3′ and 5′-ggTTTTAGAAATGCAAATTACCCAGG-3′) corresponding to a fragment of the immunoglobulin heavy-chain gene enhancer which contains an octamer binding site for POU domain proteins (22). G residues were added at the 5′ ends for labeling.

Protein-DNA binding reactions were carried out with 10 fmol of a ^{32}P end-labeled probe in a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 50 mM KCl, 10% (vol/vol) glycerol, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.05% (vol/vol) Nonidet P-40, 50 μg of bovine serum albumin (BSA), and 4 pmol of one of the two single-stranded R2 oligonucleotides. Assays with crude nuclear extracts were performed with 10 to 15 μg of protein and 0.5 μg of poly(dI-dC) · poly(dI-dC) plus, in some cases, 0.5 μg of poly(dG-dC) · poly(dG-dC). Purified proteins were assayed in the absence of a nonspecific DNA competitor. In supershift experiments, antibodies were added just before nuclear proteins. An antiserum containing Oct-1 antibodies was provided by M. Perry (The University of Texas Southwestern Medical Center, Dallas) (10). Polyclonal antibodies against Oct-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Reaction mixtures (25 to 30 μl) were incubated for 30 min at room temperature and fractionated on a 4% (wt/vol) polyacrylamide gel in 0.5× TBE buffer (45 mM Tris, 45 mM borate, 1 mM EDTA) for 3.5 to 4 h at 150 V.

Purification and characterization of RCS cell-specific DNA-binding proteins. Nuclear extracts from RCS cells (about 400 mg of protein) were diluted at 2 mg/ml in buffer A (20 mM HEPES [pH 7.9], 10% [vol/vol] glycerol, 1 mM EDTA, 0.05% [vol/vol] Nonidet P-40, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg of leupeptin and pepstatin per ml) supplemented with 70 mM NaCl. Extracts were loaded on a first 7-ml DNA affinity column which was prepared by the method described in reference 12 by covalent coupling of the wild-type double-stranded R2 oligonucleotide (see Fig. 5A) to CNBr-activated Sepharose 4B (Sigma, St. Louis, Mo.). After successive washes of the column with buffer A containing 70 and then 150 mM NaCl, chondrocyte-specific proteins were eluted in buffer A supplemented with 300 mM NaCl. The eluted fraction was diluted in 3 volumes of buffer A and loaded on a second DNA affinity column of about 6 ml. This column contained a highly mutated enhancer fragment made with complementary oligonucleotides (5′-gatccAAAGCCCGTT CTACAGCActg-3′ and 5′-aattcagATGCTGTAGAACGGGCTTTg-3′). Chondrocyte-specific proteins were eluted from this column at 150 mM NaCl. Fractions were diluted to 125 mM NaCl and loaded on a 1-ml Mono Q column (Pharmacia Biotech, Piscataway, N.J.). Chondrocyte-specific proteins were recovered in the flowthrough, which was then diluted to 50 mM NaCl and applied to a 1-ml Mono S column (Pharmacia). Proteins were eluted by using a 10-ml

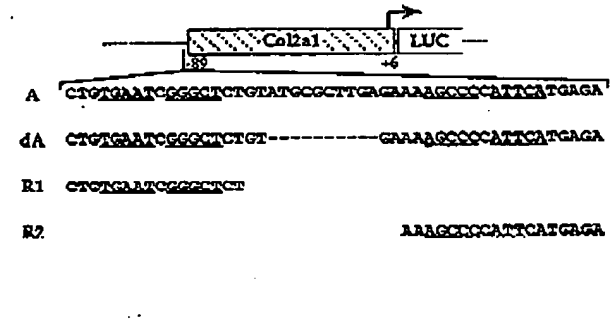


B

Intron-1 Segments	RCS	10T1/2
none	0.2 ± 0.3	0.7 ± 0.2
2 x 156	29.6 ± 6.4	2.4 ± 0.8
1 x AC	0.2	1.5
2 x AC	70.1 ± 29.7	1.6 ± 0.3
1 x AD	2.9	5.3
2 x AD	467 ± 143	5.9 ± 1.4
2 x BD	0.3	3.85
2 x A	0.0	1.8
4 x A	1478 ± 112	2.3 ± 1.0

C

```
mouse  CTGTGAATCGGGCTCTGTATGCGCTTGAGAAAAGCCCCATTCATGAGA
rat    ..........................C.......................
human  ..................G......C.......................
```



E

Intron-1 Segments	RCS (1)	RCS (2)	10T1/2
none	0.2	0.2	1.5
4 x A	1457	219	0.8
4 x dA		325	9.7
8 x R1	0.1		1.4
3 x R2		0.66	1.4
4 x R2		3.5	
6 x R2		76.9	2.3
8 x R2		469	
12 x R2		1157	4.5

FIG. 1. Delineation of an 18-bp *Col2a1* enhancer. (A) DNA constructions. The 156-bp enhancer segment of *Col2a1* intron 1 and subsegments of this enhancer, designated A, B, C, and D, were cloned individually or in combinations (indicated by dotted lines) as one copy or two or four tandem copies upstream of an 89-bp *Col2a1* promoter driving the luciferase (LUC) gene. Numbers indicate the distance of the first and last nucleotides of each segment from the transcription start site in the Col2a1 gene. (B) Transient transfection experiments with RCS and 10T1/2 cells and the constructions shown in panel A. The intron 1 segments and the number of tandem copies of these segments in each construction tested are indicated in the first column. Luciferase activities are shown as the average values ± standard deviations for two to four independent cultures tested in one or two representative experiments. (C) Nucleotide sequence of the A element. The coding strand of the mouse *Col2a1* A element is aligned with the analogous region in the human and rat genes. Only bases in the human and rat genes that differ from the mouse sequence are shown; identical bases are indicated by dots. The two inverted repeats are indicated by arrows, and their nucleotides are underlined except at one G/C mismatch. (D) Constructions made with subsegments of the A element. The sequences of subsegments dA, R1, and R2 are aligned with that of the A element. The 10-bp deletion from A in dA is represented by dashes. Nucleotides of the two repeats are underlined, except for one G/C mismatch. All elements were cloned as multiple tandem copies in the same vector as in panel A. (E) Transient transfection experiments in RCS and 10T1/2 cells. DNA constructions were made as described for panel A by using 3 to 12 tandem copies of the elements shown in panel D. Luciferase activities are shown as averages for duplicate cultures in two representative experiments with RCS cells and one experiment with 10T1/2 cells.

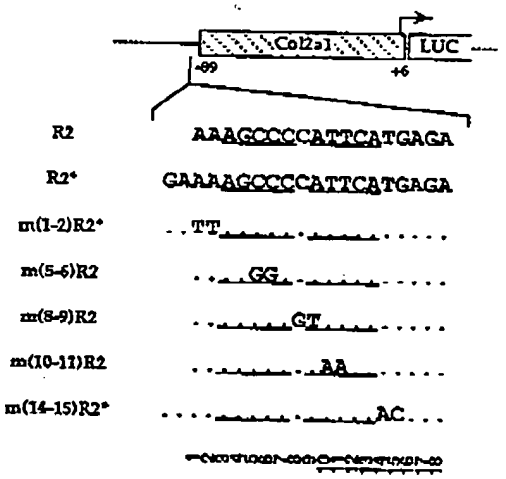
NaCl concentration gradient from 50 to 300 mM, followed by 4 ml of 1 M NaCl. Chondrocyte-specific proteins were eluted at 150 to 250 mM salt.

Protein purification was monitored by gel retardation assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 8% polyacrylamide gels. Silver staining of proteins in gels was done by the method described in reference 16, with 100 μl of Mono S fractions concentrated by precipitation with trichloroacetic acid. Protein standards were obtained from Amersham (Arlington Heights, Ill.). For protein elution-renaturation experiments, 30-μl samples of Mono S fractions were denatured at 100°C in the SDS-PAGE sample buffer. After electrophoresis, gels were extensively washed for 1 h in buffer A and cut into slices. Each slice was crushed, and proteins were eluted in 4 volumes of buffer A supplemented with 50 mM KCl and 2.5 mg of BSA per ml for 3 h at room temperature. Gel shift assays were done by adding the labeled probe to 25 μl of eluates.

RESULTS

Delineation of an 18-bp *Col2a1* enhancer in RCS cells. Results obtained in our previous studies were consistent with the notion that elements in the 5′ and 3′ parts of a 156-bp *Col2a1* intron 1 segment could be needed together to generate enhancer activity in chondrocytes (17, 28). To better delineate active enhancer elements, we divided this 156-bp segment into four subfragments (Fig. 1A). The 5′ A element contained two inverted repeats of 11 bp each with one mismatch, separated by 18 bp. This element is highly conserved among the human, rat, and mouse genes (Fig. 1C) (13, 19). The 3′ C and D elements

A



B

Intron Segments	Expt 1	Expt 2
none	0.68	0.38
12 x R2	3594	2805
12 x R2*	6050	
12 x m(1-2)R2*	1.21	
12 x m(5-6)R2		3893
12 x m(8-9)R2	0.85	
12 x m(10-11)R2	0.85	0.71
12 x m(14-15)R2*	4.68	

FIG. 2. Transcriptional activity of mutant R2 and R2* elements. The sequence of the R2 element is aligned with that of a 20-bp R2* element containing the two additional nucleotides present on the 5' side in the *Col2a1* sequence and with those of R2 and R2* mutants. The nucleotides of the R2 element were numbered 1 to 18 from the 5' to the 3' end. Only mutated nucleotides are shown; unchanged nucleotides are represented by dots. Positions of mutated nucleotides are indicated in parentheses in the designations of the mutants. Nucleotides corresponding to the 3' repeat in element A are underlined, except at a G/C mismatch. These wild-type and mutant intron 1 segments were each cloned as 12 tandem copies in the vector in Fig. 1. Constructions were transfected transiently in RCS cells. Luciferase activities are presented as averages for two (experiment 1) or three (experiment 2) cultures in representative experiments. Note that the R2* element appears to be more active than the R2 element in experiment 1 but on average the two elements were similarly active. LUC, luciferase.

each corresponded to a sequence that showed DNase I protection with nuclear extracts of both RCS cells and 10T1/2 fibroblasts (17). The B element partially overlaps A and C. Various combinations of these elements were cloned upstream of an 89-bp *Col2a1* promoter driving the luciferase reporter gene, and the constructions were tested in transient transfections in RCS and 10T1/2 cells (Fig. 1A and B). As observed previously (17), the promoter by itself was barely active in the two cell lines, but it was highly activated in RCS cells and only minimally activated in 10T1/2 fibroblasts by two copies of the 156-bp enhancer. One copy of either AC or AD was essentially inactive in both cell types, but two copies of either combination strongly stimulated promoter activity in RCS cells but not in fibroblasts (Fig. 1B). Two copies of BD and two copies of A alone were essentially inactive in both cell types. Four tandem copies of A alone induced very strong promoter activation in RCS cells, reaching a level about 50-fold higher than that achieved by two copies of the 156-bp enhancer. Activation was minimal in fibroblasts. We concluded that the 48-bp A element contained the *cis*-acting sequences responsible for the RCS cell specificity of the *Col2a1* enhancer and that the C and D segments might contain binding sites for proteins that cooperated with factors binding to A in order to generate a high level of enhancer activity in RCS cells. Although D was more potent than C, these two segments appeared to play similar roles and were not necessary together.

We then tested whether the two inverted repeats in A and their 18-bp linker were necessary for enhancer activity (Fig. 1D and E). Four copies of an element harboring a 10-bp deletion within the linker (dA) activated the 89-bp *Col2a1* promoter in RCS cells as efficiently as A. Eight copies of an element containing the 5' repeat (R1) failed to activate the promoter in either RCS cells or 10T1/2 cells. However, an element containing the 3' repeat (R2) increased promoter activity in RCS cells in a copy-number-dependent manner, reaching several thousand-fold with 8 and 12 copies. Very little activation was detected in 10T1/2 cells with multiple copies of R2. Multiple copies of an 18-bp sequence containing the 3' repeat therefore appeared sufficient to induce high levels of promoter activation in RCS cells.

Abolition of the activity of the 18-bp enhancer by specific mutations. To delineate the binding site for DNA-binding proteins potentially implicated in enhancer activity in RCS cells, constructions in which transversion mutations were introduced in the 18-bp R2 sequence or in a 20-bp R2* sequence, which contains two additional nucleotides at the 5' end, were made (Fig. 2). Twelve copies of the R2 and R2* elements were similarly active in RCS cells (Fig. 2). Mutation of the nucleotide pair 5-6 slightly increased the activity of the enhancer in RCS cells, whereas mutation of the nucleotide pair 1-2, 8-9, 10-11, or 14-15 abolished activity. These results indicated that nucleotides both inside the repeat (8-9 and 10-11) and in the 5' (1-2) and 3' (14-15) flanking sequences were essential for enhancer activity. The importance of nucleotides located outside the repeat is in agreement with the absence of activity of R1, whose nucleotides flanking the repeat are different from those in R2.

Activity of *Col2a1* enhancer fragments in primary chondrocytes. The activities of *Col2a1* enhancer elements were tested in mouse rib chondrocytes by transfecting primary cells soon after their isolation from cartilage when they were still fully differentiated (14). The 89-bp *Col2a1* promoter was barely active in these cells but was strongly activated by *Col2a1* enhancer fragments that were active in RCS cells (Table 1). These included a two-copy 231-bp element, the 4-copy 48-bp A element, and the 12-copy 20-bp R2* element. Similar results were obtained with the 12-copy R2 and R2* elements (data not shown). The 231-bp element, previously shown to be a strong enhancer in RCS cells (17), contained the 156-bp enhancer plus 75 bp of the 5' upstream sequence. Mutations in the 18-bp enhancer that abolished activity in RCS cells did the same in primary chondrocytes (Table 1). Hence, the minimal *Col2a1* enhancer elements were active in primary chondrocytes as well as in RCS cells.

TABLE 1. Transfection of primary chondrocytes with Col2a1 constructions

Intron segment[a]	Luciferase activity[b]
None	0.31 ± 0.16
2 × 231	271 ± 77
4 × A	39.5 ± 1.5
12 × R2*	153 ± 19
12 × m(1-2)R2*	0.98 ± 0.17
12 × m(14-15)R2*	0.50 ± 0.30

[a] The 89-bp *Col2a1* promoter construction (Fig. 1) was tested in parallel with constructions containing *Col2a1* intron 1 segment 2 × 231 (two tandem copies of a 231-bp enhancer segment spanning nucleotides +2113 to +2343 of the Col2a1 gene) or 4 × A (four copies of the A element [+2188 to +2234]) (Fig. 1) or one of the segments in Fig. 2.

[b] Average ± standard deviation for triplicate cultures of one representative experiment.

Activity of *Col2a1* enhancer fragments in transgenic mice. To verify the chondrocyte specificity of the minimal enhancer elements in vivo, we generated transgenic mice harboring four copies of A cloned in the vector used in our previous study with longer *Col2a1* enhancer fragments (28), in which a 309-bp *Col2a1* promoter drove the βgeo reporter gene (Fig. 3A). This promoter was unable by itself to direct cartilage expression (28). Two of three transgenic founder embryos collected 14.5 days postcoitus stained positively with X-Gal, a chromogenic substrate for β-galactosidase, whereas the third embryo showed no staining. The pattern of staining in the positive embryos was similar to the one obtained with longer enhancer fragments (28), although it was somewhat less intense. Whole-mount embryos showed staining in the cartilages of the ear and nose, in the cartilage anlagen of the limb long bones, in pelvic and shoulder girdles, and in vertebrae and ribs (Fig. 3B). Histological analysis of multiple sections throughout the whole embryos indicated that only chondrocytes stained with X-Gal (Fig. 3C). The A element thus appeared to be sufficient to confer chondrocyte-specific expression in vivo.

Of six transgenic mouse embryos shown by Southern analysis to harbor 12 copies of the R2 element cloned in the same vector, none stained with X-Gal (data not shown). Since the luciferase assay is more sensitive than X-Gal staining, transgenic mice were generated with the same construction that was used in transfections, i.e., with 12 copies of R2 cloned upstream of the 89-bp *Col2a1* promoter, itself linked to the luciferase gene. Significant luciferase activity was detected in newborn transgenic mice in extracts from rib cages and vertebrae which contained cartilage, from the brain and skin, and from the tail which contained both cartilage and skin besides other tissues (Table 2). All other nonchondrogenic organs were negative. Chondrocytes isolated from the ribs of transgenic mice contained high levels of luciferase activity (Table 2). Hence, although the construction allowed promiscuous expression in some nonchondrogenic tissues, our results indicated that the R2 element was able to direct promoter expression in chondrocytes in vivo.

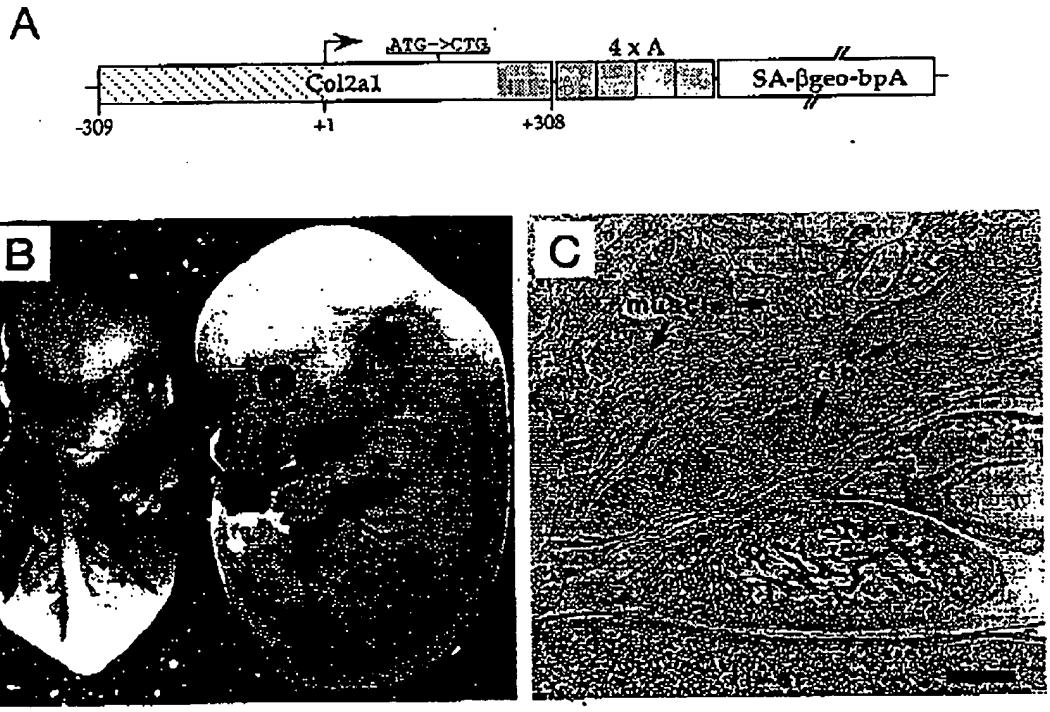


FIG. 3. X-Gal staining of transgenic mice harboring four copies of element A. (A) Schematic representation of the DNA construction. Four tandem copies of the A element were inserted upstream of the SA-βgeo-bpA cassette and downstream of a *Col2a1* segment extending from −309 to +308. The SA-βgeo-bpA cassette includes a splice acceptor (SA), which allows correct splicing of the intron sequence; the βgeo gene, which encodes a fusion protein with *Escherichia coli* β-galactosidase and neomycin resistance activities; and the bovine growth hormone polyadenylation signal (bpA). The *Col2a1* segment contains 309 bp of promoter sequences, exon 1, and the proximal 70 bp of intron 1. The *Col2a1* translation initiation codon was mutated to CTG to favor translation initiation at the AUG codon of βgeo RNA. (B) Frontal and lateral views of two different transgenic founder embryos stained with X-Gal at 14.5 days postcoitus. (C) Histological analysis of a portion of a sagittal section of one of the two embryos shown in panel B. The section was counterstained with eosin. Bar, 400 μm.

TABLE 2. Luciferase activities in tissue extracts from transgenic mice harboring 12 copies of the R2 element[a]

Extract	Activity[b]		
	Mouse 1	Mouse 2	Mouse 3
Rib cage	173	27	19
Vertebrae	88	28	9
Brain	114	167	18
Skin	154	7	7
Tail	430	20	383
Calvarium			3
Muscle	7	1	2
Lung	3	0	0
Heart	5	0	1
Liver	1	0	0
Intestine	5	0	0
Spleen	1	0	5
Kidney	1	0	0
Thymus		2	2
Tail[c]	245	10	8
Chondrocytes[c,d]	1,478	250	392

[a] Transgenic mice were generated with a construction made of 12 copies of the R2 element cloned upstream of the 89-bp *Col2a1* promoter driving the luciferase gene (Fig. 1). Founder mice expressing the transgene were sacrificed within 3 days after birth, and luciferase activities in the indicated tissue extracts were measured.

[b] In relative luminescence units per microgram of protein.

[c] The last two rows of data correspond to three mice different from the three mice whose data are listed in the rest of the table.

[d] Isolated from the rib cages of transgenic mice harboring the same construction as above. Luciferase activity was measured directly after digestion of rib cartilages by collagenase and extensive washes of the cells in phosphate-buffered saline.

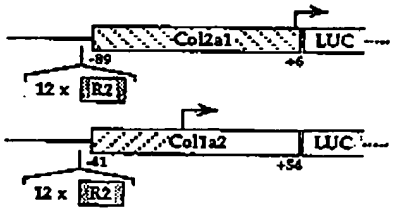
Specific activation of a heterologous promoter by the 18-bp enhancer. We showed previously that the *Col2a1* promoter does not contain elements necessary for chondrocyte expression, using constructions in which a 231- or 182-bp *Col2a1* enhancer fragment was cloned upstream of either a minimal adenovirus major late promoter or a minimal β-globin promoter (17, 28). This result was confirmed with a construction containing the 12-copy R2 element cloned upstream of a minimal promoter (−41 to +54) of the mouse proα2(I) collagen gene (*Col1a2*) (Fig. 4). This short promoter, which contains no activating elements upstream of the TATA box (8), was essentially inactive in RCS and 10T1/2 cells, but it was significantly activated in RCS cells, not in fibroblasts, by the 12-copy 18-bp enhancer (Fig. 4). The level of activation of the minimal *Col2a1* promoter was, however, lower than that of the 89-bp *Col2a1* promoter. It is thus possible that the 89-bp *Col2a1* promoter contains elements not present in the shorter *Col2a1* promoter that support transactivation by the R2 multimer.

Proteins selectively expressed in RCS cells and primary chondrocytes bind a discrete sequence in the 18-bp enhancer. To determine whether RCS cells and chondrocytes express unique nuclear factors that specifically bind to the 18-bp enhancer and to locate the precise DNA binding sites of these factors, gel retardation assays were performed with nuclear extracts from various cell types and oligonucleotide probes containing the R2 element in its wild-type form and a series of mutant forms (Fig. 5A). These mutant probes each contained two different adjacent nucleotides modified by transversion.

Six major DNA-protein complexes were separated by electrophoresis after incubation of RCS cell nuclear extracts with the wild-type probe (Fig. 5B). Complexes 1, 2, 5, and 6 likely corresponded to ubiquitous proteins since they were formed with nuclear extracts from most cell types. Complex 3 was formed with nuclear extracts from RCS cells and was the major complex observed with extracts from primary chondrocytes. A complex with approximately similar mobility was also seen with nuclear extracts from the lymphoma BL-4 and Raji cell lines, but it was absent in nuclear extracts from fibroblast cell lines (10T1/2 and 7f4 cells), ROS osteosarcoma cells, and all other cell lines tested (C_2C_{12} myoblasts, HeLa cervical carcinoma cells, S194 mycloma cells, and NMuLI liver cells). Complex 4 was formed exclusively by nuclear extracts from RCS cells. We tentatively concluded that complex 3 contained one or several proteins selectively expressed in RCS cells and primary chondrocytes, whereas complex 4 contained one or several proteins present exclusively in RCS cells.

Oligonucleotide probes containing mutations in nucleotides 8 to 17 were unable to efficiently form complexes 3 and 4 or complex 1 (Fig. 5C). These included probes with mutations in the nucleotide pairs 8-9, 10-11, and 14-15 which were shown to abolish chondrocyte-specific enhancer activity completely (Fig. 2 and Table 1). In contrast, mutation of the nucleotide pair 1-2 which was also shown to abolish enhancer activity did not significantly affect the formation of any DNA-protein complex, nor did mutation of the nucleotide pair 3-4. Mutations of nucleotides 6 and 7 allowed stronger binding of proteins in complexes 3 and 4, and also complex 1, an effect which could be related to the slightly higher enhancer activity of the R2

A



B

Constructions	RCS	10T1/2
p89Col2a1	0.56	5.59
12 x R2 - p89Col2a1	1406	4.19
p41Col1a2	0.06	0.86
12 x R2 - p41Col1a2	18.8	0.64

FIG. 4. Activation of a heterologous promoter by 12 copies of the R2 element. RCS and 10T1/2 cells were transfected transiently with the following constructions. p89Col2a1 contained the 89-bp *Col2a1* promoter cloned upstream of the luciferase (LUC) gene in pLuc4 (Fig. 1); p41Col1a2 contained a 41-bp *Col2a1* promoter (−41 to +54) cloned upstream of the luciferase gene in pLuc4; 12xR2-p89Col2a1 and 12xR2-p41Col1a2 were made by cloning 12 copies of the R2 element directly upstream of the promoter in p89Col2a1 and p41Col1a2, respectively. Luciferase activities are averages for duplicate cultures in one representative experiment.

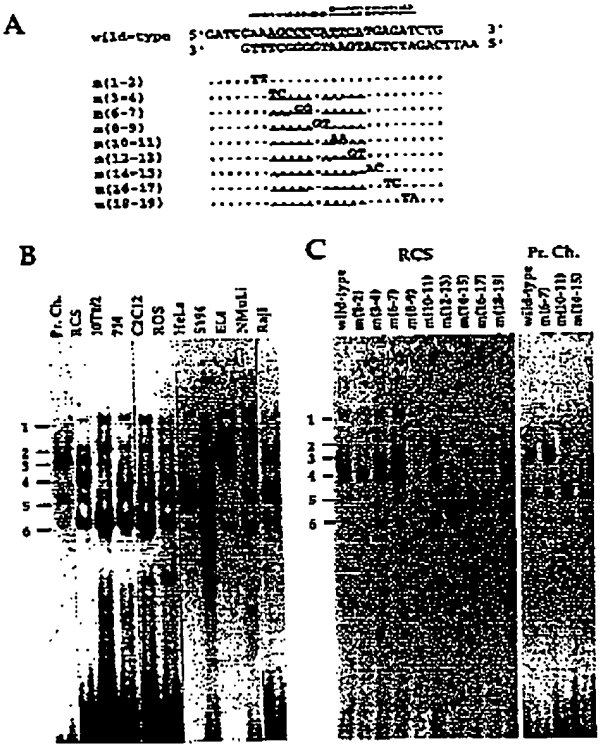


FIG. 5. Identification of chondrocyte-specific proteins binding to the R2 element. (A) Oligonucleotide probes used in gel retardation assays. The wild-type probe consisted of a double-stranded oligonucleotide corresponding to the 18-bp R2 element to which nucleotides were added to form a *Bam*HI restriction site at the 5′ end and a *Bgl*II restriction site followed by an *Eco*RI restriction site at the 3′ end. The nucleotides of the R2 sequence were numbered 1 to 18 from the 3′ to the 3′ side of the coding strand; number 19 was given to the proximal nucleotide of the 3′ flanking sequence. Mutant probes are designated by the letter m followed by the mutated nucleotides in parentheses. These probes were identical to the wild-type probe except for the mutated nucleotides. Only the coding strand is shown, with dots indicating nucleotides identical to those in the wild-type sequence. Nucleotides forming the 3′ repeat of element A are underlined. (B) Gel retardation assay with the R2 wild-type probe and nuclear extracts from various cell types. The following cell types were used: primary mouse rib chondrocytes (Pr. Ch.), RCS cells, 10T1/2 mouse embryo fibroblasts, subline 714 of BALB/3T3 mouse embryo fibroblasts, C_2C_{12} mouse skeletal myoblasts, ROS 17/2.8 rat osteosarcoma cells, HeLa human carcinoma epithelioid cells, S194 mouse myeloma cells, EL4 mouse lymphoma T-type cells, NMuLi mouse normal liver cells, and Raji human lymphoblast-like cells. Six major DNA-protein complexes, numbered 1 to 6, were separated by electrophoresis after incubation of the RCS cell nuclear extracts with the R2 probe. (C) Gel retardation assays with R2 mutant probes and nuclear extracts from RCS cells and primary chondrocytes. The major protein-DNA complexes formed by incubation of RCS cell nuclear extracts with the wild-type probe are indicated. Bands 2, 5, and 6 appear fainter in the RCS samples in panel C than in panel B because of the addition of 0.5 μg of poly(dG-dC) · poly(dG-dC) to the reaction mixtures. Note that bands 3 and 4 appear weaker with the m(1-2) probe than with the wild-type probe, but this was not the case in other experiments.

element that contained mutations in nucleotides 5 and 6. Finally, mutations of nucleotides 18 and 19 (nucleotide 19 is part of the flanking sequence added to the 18-bp element and does not correspond to the Col2a1 sequence) did not affect the formation of any DNA-protein complex. The proteins in complexes 1, 3, and 4 thus appeared to bind to the same sequence, CATTCATGAG, suggesting that these proteins might belong to the same family of DNA binding factors.

Only the 5′ part of this 10-bp binding site is conserved in the R1 element. In agreement with this partial homology, complexes 1, 3, and 4 were not observed when the R1 element was used as a probe in gel retardation experiments with nuclear extracts of RCS cells (data not shown). When the R1, R2, and mutant R2 oligonucleotides were used in competition with the R2 wild-type probe, results were consistent with those obtained in direct binding experiments, i.e., only the R1 oligonucleotide and the R2 oligonucleotides with mutations in nucleotides 8 to 17 were unable to compete for the formation of complexes 1, 3, and 4, whereas the R2 oligonucleotide competed efficiently (data not shown).

These results showed that proteins with a restricted pattern of cellular expression were present in RCS cells and primary chondrocytes and bound to the 18-bp enhancer. All mutations within the binding site for these proteins that were functionally tested in transfection experiments abolished enhancer activity, strongly suggesting a role of these proteins in enhancer activity. These results, however, do not explain why a mutation of the two nucleotides located at the 5′ end of the 18-bp enhancer abolished activity since no significant difference was seen in gel retardation assays whether a wild-type probe or a probe mutated in these two nucleotides was used. It is possible that these nucleotides were too close to the 5′ end of the oligonucleotide and therefore could not bind proteins efficiently. When larger oligonucleotides that extended more upstream were used, larger DNA-protein complexes were observed with extracts of RCS cells, but these complexes could not be distinguished from those obtained with extracts from 10T1/2 fibroblasts (data not shown).

The two chondrocyte-specific enhancer-binding proteins exhibit similar biochemical properties. In order to better characterize the chondrocyte-specific enhancer-binding proteins present in complexes 3 and 4, these proteins were extensively purified from RCS cell nuclear extracts by sequential chromotographies through two different DNA affinity columns, followed by Mono S and Mono Q ion-exchange columns (see Materials and Methods). The proteins present in these two complexes copurified through the four columns (data not shown). The Mono S fractions containing these proteins (Fig. 6A) were then fractionated by SDS-PAGE. Silver staining of the gel showed only a few protein species (Fig. 6B). The two most intense bands corresponded to proteins with apparent M_rs of 52,000 and 54,000. The relative intensity of these two bands and their Mono S elution profile were consistent with the hypothesis that the upper and lower bands in the SDS-PAGE corresponded, respectively, to complexes 3 and 4 in gel shift assays. DNA-binding experiments performed after elution-renaturation of proteins eluted from gel slices cut from another lane of the same gel as the one used for silver staining confirmed that the 54- and 52-kDa proteins formed, respectively, complexes 3 and 4 in gel shift assays (Fig. 6C and D). Gel retardation assays performed with purified proteins (Mono S eluates) and mutant R2 probes confirmed that the purified proteins were binding DNA to the 10-bp site identified with crude extracts (data not shown). Furthermore, methylation interference assay using purified proteins and a DNA probe containing the 48-bp A enhancer element also indicated that these proteins contacted DNA in the 10-bp site (data not shown).

These results indicated that the two chondrocyte-specific DNA-binding proteins have similar M_rs and must be closely related since they copurified and showed similar DNA-binding properties. Since each one of the two DNA-binding activities was recovered from gel slices that contained a single silver-stained band, it appears likely that the proteins bound DNA as monomers or as homodimers.

The chondrocyte-specific enhancer-binding proteins are likely POU domain proteins. A search in the Findpatterns database of the Genetics Computer Group (University of Wis-

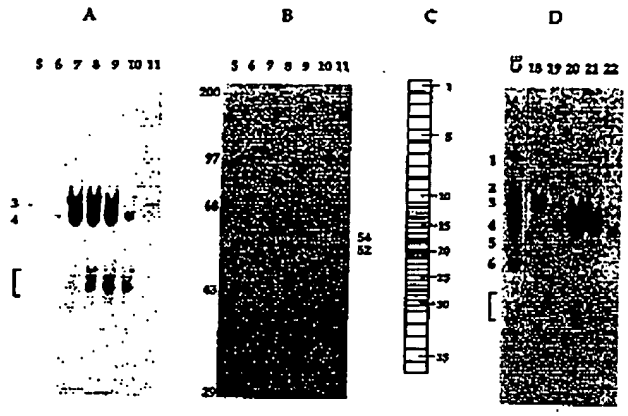


FIG. 6. Characterization of a purified preparation of chondrocyte-specific nuclear proteins. The cell-specific enhancer-binding proteins present in nuclear extracts from RCS cells were purified by chromatography through two different DNA affinity columns and then through Mono Q and Mono S columns, as described in Materials and Methods. The experiments in panels A to D were all performed with the same Mono S fractions. (A) Gel shift analysis. Mono S fractions were tested in gel retardation assay using the R2 probe. Only fractions 5 to 11 which contained the peak of activity of complexes 3 and 4 are shown. The micromolar salt concentrations of these fractions were as follows: 5, 122; 6, 147; 7, 176; 8, 202; 9, 233; 10, 252; 11, 275. A bracket indicates complexes of higher electrophoretic mobility which likely correspond to partially degraded purified proteins. No probe shift was detected in the flowthrough of the chromatography or in other elution fractions (data not shown). (B) Silver staining of proteins eluted in the Mono S fractions 5 to 11 and fractionated by SDS-PAGE. The M_rs of protein standards are indicated on the left, and the M_rs of two major protein species are indicated on the right. (C) Schematic representation of gel slicing. A pool of fractions 7 to 9 from Mono S chromatography was loaded on the same gel as in panel B. After electrophoresis and gel washes, the lane of the gel that contained the sample was cut into horizontal slices which were numbered as indicated. Shaded boxes represent gel slices 18, 20, and 21, from which proteins forming complexes 3 and 4 were recovered (see panel D). (D) Gel shift assay with proteins eluted from SDS-PAGE. Proteins eluted from the gel slices shown in panel C were tested in gel shift assay using the R2 probe. Only the assays for slices from which proteins forming complexes 3 and 4 were recovered are shown. Slice numbers are indicated at the top. Crude extracts (CE) from RCS cells were used as a standard, and complexes 1 to 6 formed with these extracts are indicated.

consin, Madison) indicated that the sequence of the 10-bp DNA-binding site, CATTCATGAG, might bind members of the POU domain protein family of transcription factors. This sequence contains a low-affinity heptamer consensus binding site (CTCATGA) on its lower strand and an overlapping imperfect high-affinity octamer binding site for these proteins on its upper strand (ATTAATGC). A characteristic of POU domain proteins is their flexibility in DNA sequence recognition (9). We, therefore, investigated whether the chondrocyte-specific DNA-binding proteins might be POU domain proteins.

We performed gel shift assays using in parallel the wild-type R2 enhancer probe (R2) and a probe (OCT) containing a consensus octamer binding site for POU domain proteins. Three major DNA-protein complexes, 1*, 3*, and 4*, were obtained after incubation of RCS cell nuclear extracts with the OCT probe (Fig. 7A, lanes 11 and 16). The electrophoretic mobilities of these complexes were similar to those of complexes 1, 3, and 4 formed with the R2 probe (lanes 1 and 6). The OCT oligonucleotide competed very efficiently for the formation of complexes 1, 3, and 4 with labeled R2, at least 10 times better than unlabeled R2 itself (compare lanes 1 to 5 with lanes 6 to 10); accordingly, R2 competed less efficiently for the binding of proteins to the OCT probe than unlabeled OCT (compare lanes 11 to 15 with lanes 16 to 20). These results indicated that the proteins forming complexes 1, 3, and 4 with R2 were likely the same as those forming complexes 1*, 3*, and 4*, respectively, with OCT. Furthermore, these proteins likely belonged to the POU domain protein family since they bound with a higher affinity to the OCT oligonucleotide that contains a strong binding site for POU domain proteins than to the low-affinity and imperfect binding sites for these proteins in R2. Nuclear extracts from primary chondrocytes formed two major complexes with OCT, which corresponded to complexes 1* and 3* from RCS cell nuclear extracts (data not shown). Complex 3* was much more abundant than complex 1*, and, similarly, complex 3 formed with R2 was much more abundant than complex 1. These results further suggest that the specific proteins identified in nuclear extracts from RCS cells and primary chondrocytes were identical. The specific proteins purified from RCS cells also bound very efficiently to OCT (Fig. 7B), confirming that the proteins that bound to the two probes were identical.

Since complex 1 was formed with a protein widely expressed (Fig. 7A) and since Oct-1 is a ubiquitous POU domain protein, we asked whether complex 1 contained Oct-1. An antiserum containing antibodies against Oct-1 produced a supershift of complexes 1 and 1* formed with nuclear extracts from RCS cells (Fig. 7C) and from other cell types that were tested (data not shown), with both the R2 probe and the OCT probe, thus confirming that complex 1 contained Oct-1.

Antibodies against the predominantly lymphoid-restricted Oct-2 appeared to supershift the complex formed with EL-4 and Raji nuclear extracts that had approximately the same mobility as complexes 3 and 3*, but these antibodies did not supershift complex 3* or complex 4*, formed either with RCS cell nuclear extracts (Fig. 7D) or with purified proteins from RCS cell extracts (data not shown). These results strongly suggested that the protein present in complex 3* formed with chondrocyte extracts very likely corresponds to another POU domain protein.

Altogether, our DNA binding experiment data strongly suggest that RCS cells and primary chondrocytes express one or two members of the POU domain protein family which bind to a 10-bp sequence in the minimal *Col2a1* enhancer element. We hypothesize that these proteins play a role in enhancer activity.

Abolition of the activity of 465- and 231-bp enhancer elements by deletion of the 10-bp binding site for chondrocyte-specific proteins. In order to determine whether the 10-bp sequence which binds chondrocyte-specific proteins and is essential for the enhancer activity of the multimerized 18-bp sequence in chondrocytes was also essential for the activity of larger intron 1 fragments (17, 28), we tested the activities of enhancer segments of 465 and 231 bp in which these 10 bp were deleted (Fig. 8). Whereas the 465-bp wild-type segment present as a single copy stimulated promoter activity in RCS cells, the (465 − 10)-bp element was inactive (Fig. 8A). Similarly, the 231-bp wild-type fragment stimulated promoter activity in RCS cells when tested as one copy and about 10 times more than that when tested as two tandem copies, but constructions containing either one copy or two copies of the (231 − 10)-bp fragment showed no significant enhancer activity in RCS cells (Fig. 8B). The 465-bp fragment was inactive in 10T1/2 fibroblasts, but, interestingly, the (465 − 10)-bp fragment slightly increased promoter activity in these cells (Fig. 8A). The wild-type 231-bp segment modestly increased promoter activity in 10T1/2 cells, but a similar level of activation was obtained with one and two copies. The 10-bp deletion did not affect or slightly stimulated the promoter activation induced by, respectively, one copy or two copies of the 231-bp segment (Fig. 8B). Although the higher level of promoter activation obtained in fibroblasts with constructions containing the 10-bp deletion was reproducible (data not shown), its sig-

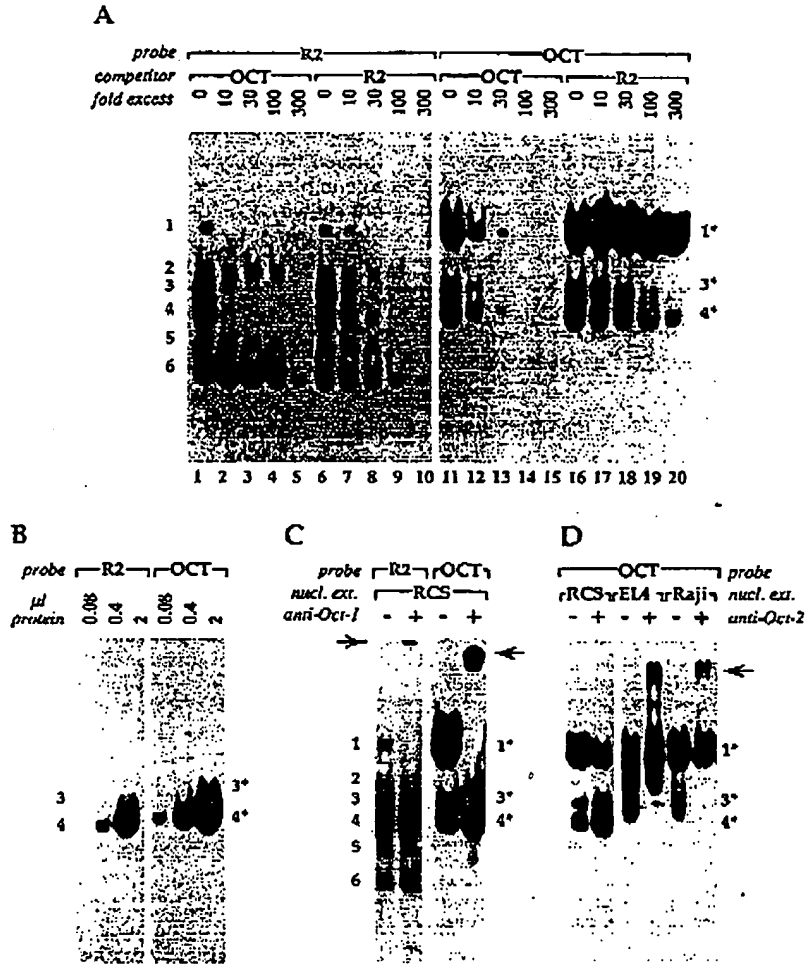


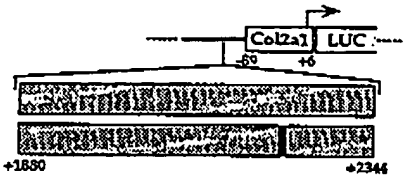
FIG. 7. Gel shift analyses using the R2 and OCT probes and antibodies against Oct-1 and Oct-2. (A) Direct binding and competition assays. Nuclear extracts from RCS cells were incubated with the R2 or OCT probe and with a 0- to 300-fold excess of an unlabeled competitor probe, as indicated. The major complexes obtained with the R2 probe are labeled 1 to 6, and the major complexes obtained with the OCT probe are labeled 1*, 3*, and 4*. (B) DNA-binding assays with purified proteins. Increasing amounts of a purified preparation of RCS cell-specific nuclear proteins (0.08, 0.4, and 2 μl) were incubated with the R2 or OCT probe. Note that the preparation used in this experiment was enriched in protein forming complex 4 relative to protein forming complex 3; in other experiments, the purified protein forming complex 3 with the R2 probe also bound tightly to the OCT probe. (C) Supershift of complexes 1 and 1* with antibodies directed against Oct-1. RCS cell nuclear extracts (nucl. ext.) were incubated with the R2 or OCT probe, in the presence (+) or absence (−) of 2 μl of antiserum containing antibodies against Oct-1. Supershifts are indicated with arrows. No supershift was observed with a nonimmune serum (data not shown). (D) Supershift of Oct-2 with specific antibodies. Nuclear extracts from RCS, EL4, and Raji cells were incubated with the OCT probe in the presence (+) or absence (−) of antibodies directed against Oct-2. Supershifts are indicated with an arrow.

nificance is not understood. It is possible that the deletion created a binding site for transcriptional activators present in fibroblasts that would otherwise never be allowed to play a role in the context of a wild-type enhancer. Alternatively, it is possible that fibroblasts contain proteins that bind to the wild-type enhancer, not the deleted version, and that decrease the low degree of activity of the chondrocyte enhancer in fibroblasts. These results nevertheless indicated that the 10-bp binding site for chondrocyte-specific proteins was involved in the high-level chondrocyte-specific activity of the *Col2a1* intron 1 enhancer.

DISCUSSION

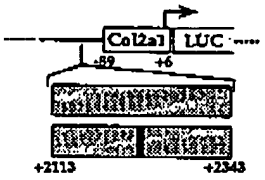
We have delineated a minimal 18-bp sequence in the first intron of the Col2a1 gene which after multimerization enhanced promoter activity several thousand-fold in RCS cells and primary chondrocytes and only minimally in fibroblasts. This multimerized enhancer also generated promoter expression in chondrocytes of transgenic mice. The 18-bp sequence is part of a DNA motif which is highly conserved between the human, rat, and mouse genes and which is made up of two inverted repeats of 11 bp each, separated by an 18-bp linker. The repeats themselves contain no consensus binding site for known DNA-binding proteins, and their significance is not yet understood. The 18-bp enhancer includes the 3′ repeat and flanking nucleotides. Mutational analysis has shown that nucleotides both inside the repeat and in the 5′ and 3′ flanking sequences were essential for enhancer activity. Interestingly, an element that included the 5′ repeat and flanking nucleotides was inactive. This result further emphasized the role in enhancer activity of nucleotides adjacent to the 3′ repeat since the nucleotides flanking the two repeats are different.

A



Intron 1 Segments	RCS	10T1/2
none	0.01	0.09
465 bp	0.56	0.03
(465-10) bp	0.04	0.20

B



Intron 1 Segments	RCS	10T1/2
none	0.01	0.13
1 x 231 bp	2.17	0.78
1 x (231-10) bp	0.03	0.64
2 x 231 bp	20.7	0.86
2 x (231-10) bp	0.08	1.67

FIG. 8. Effect of a 10-bp deletion in large intron 1 fragments on enhancer activity. (A) Deletion within a 465-bp intron 1 segment. DNA constructions were made similarly to those in Fig. 1 by using as a tester enhancer either a 465-bp intron 1 fragment (spanning nucleotides +1880 to +2344 in the Col2a1 gene) or a corresponding intron 1 segment [(465-10) bp] in which the 10-bp binding site for chondrocyte-specific proteins had been deleted (nucleotides +2224 to +2233 [solid portion]). These constructions and a construction containing the promoter only were transfected transiently in RCS and 10T1/2 cells. Luciferase activities are shown as averages for duplicate cultures in one representative experiment. (B) Deletion in a 231-bp intron 1 segment. Constructions similar to those used in panel A were made by using either a 231-bp intron 1 enhancer fragment (+2113 to +2343) or a corresponding intron 1 segment containing the same 10-bp deletion as in the 465-bp segment. These segments were cloned as one copy (1×) or as two tandem copies (2×), and the constructions were transfected transiently in RCS and 10T1/2 cells. Luciferase activities are averages for duplicate cultures of one representative experiment.

Gel retardation assays have indicated that nuclear proteins selectively expressed in RCS cells and primary chondrocytes bind to a 10-bp sequence located at the 3′ end of the 18-bp enhancer. Point mutations within this site both abolished the binding of these proteins and blocked the activity of the multimerized 18-bp enhancer. Moreover, deletion of these 10 bp in intron 1 segments of 465 and 231 bp resulted in essentially complete loss of enhancer activity in RCS cells. These results therefore indicated that this 10-bp *cis*-acting element plays a central role in the activity of the chondrocyte enhancer and strongly suggest that the chondrocyte-specific proteins are key players in the activation of the Col2a1 gene in chondrocytes.

One of the chondrocyte-specific proteins was present exclusively in RCS cell nuclear extracts. The other one was present both in RCS cells and in primary chondrocytes but in no other cell type tested. A computer search indicated that the sequence of the 10-bp protein binding site contains a low-affinity heptamer binding site for members of the POU domain family of transcription factors and might also contain an overlapping imperfect high-affinity octamer binding site for these factors. Several lines of evidence that the chondrocyte-specific proteins might indeed belong to this family were obtained. The proteins bound with higher affinity to a probe containing a consensus octamer binding site for POU domain proteins than to the 18-bp Col2a1 probe. Antibodies directed against Oct-1, a ubiquitous POU domain protein, supershifted a complex that was formed with all nuclear extracts tested and that presented DNA binding properties similar to those of the chondrocyte-specific proteins. Elution-renaturation experiments indicated that the chondrocyte-specific proteins bound DNA either as monomers or as homodimers, a result in agreement with the ability of POU domain proteins to bind DNA as monomers. Since no POU domain protein is known to be expressed specifically in cartilages, one can speculate that chondrocytes express still unknown members of this family. Although Oct-1 is ubiquitously expressed and involved in the expression of housekeeping and cell-specific genes, several other POU domain proteins show a cell-type-restricted pattern of expression and have been implicated in tissue-specific expression of various genes (9). Analytical purification indicated that the two chondrocyte proteins were closely related since they copurified through several DNA affinity and ion-exchange chromatographies. Moreover, their apparent M_rs differ by only 2,000. It is possible therefore that they represent two different products of one gene. Large-scale purification of the proteins will be necessary to obtain partial amino acid sequences, knowledge of which would then be used to isolate cDNAs.

Our mutational and deletion analyses also indicated that besides the 10-bp binding site for chondrocyte-specific proteins multiple other sequences present in *Col2a1* intron 1 and the promoter were important for enhancer activity. These other elements might bind proteins that cooperate with each other and with the chondrocyte-specific proteins in order to achieve high-level promoter activation. One such element must exist at the 5′ end of the 18-bp enhancer since a mutation of the two most-5′ nucleotides abolished enhancer activity. Other elements are probably present in the 48-bp element outside the 18-bp sequence since four copies of the 48-bp element were able to enhance promoter activity in chondrocytes at a much higher level than four copies of the 18-bp element. We have also demonstrated that the 3′ part of the 156-bp intron 1 enhancer fragment harbored two sequences each of which potentiated the activity of the 48-bp enhancer. We have shown previously that these sequences were similarly footprinted by nuclear extracts from 10T1/2 fibroblasts and RCS cells, suggesting that they might bind ubiquitous proteins (17). Along

with the progressive truncation of *Col2a1* intron 1 performed to delineate elements involved in the chondrocyte specificity of the enhancer, it appeared that more and more copies of active fragments were gradually necessary to generate promoter activation. Multimerization of short elements might compensate for the progressive deletion of elements important for enhancer strength in much larger DNA segments. Cooperation between enhancer-binding proteins and proteins binding to the 89-bp *Col2a1* promoter could also be implicated in enhancer strength since the level of promoter activation obtained with a minimal *Col2a1* promoter was much lower than with the 89-bp *Col2a1* promoter. It can be mentioned here that promoter activation was specific to RCS cells regardless of whether a *Col2a1* or *Col2a1* promoter was used, confirming our previous observation that the *Col2a1* promoter is not required for chondrocyte expression (17, 28). In agreement with our results, Savagner and collaborators recently showed that elements located within the proximal promoter of the *Col2a1* gene were required for high-level expression of constructions containing a *Col2a1* intron 1 enhancer segment in chicken embryo chondrocytes (20).

In transgenic mice, four copies of the 48-bp enhancer conferred high levels of chondrocyte expression on a *lacZ* reporter gene. Twelve copies of the 18-bp enhancer cloned in the same vector could not activate this promoter to levels sufficient to detect staining of chondrocytes by X-Gal. However, when 12 copies of the 18-bp enhancer were cloned upstream of a promoter driving the luciferase gene, promoter activity was detected by the very sensitive luciferase assay in cartilage tissues and chondrocytes isolated from newborn mice and not in most other tissues. Since 4 copies of the 48-bp element were less active than 12 copies of the 18-bp sequence, it is possible that the 48-bp enhancer contains DNA elements which bind a factor(s) active in opening the chromatin in vivo, eventually in conjunction with chondrocyte-specific protein(s) binding to the 18-bp element, and that these elements would have been deleted when the enhancer was shortened to 18 bp.

In addition to expression in cartilages, the construction containing 12 copies of the 18-bp enhancer cloned upstream of the 89-bp *Col2a1* promoter was also active in the skin and brain in newborn transgenic mice. Similarly, we had previously shown that some expression in the brain was present in addition to cartilage expression in transgenic mice harboring a 182-bp intron 1 fragment cloned upstream of a minimal β-globin promoter (28). We do not know whether this construction was expressed in the skin since transgenic embryos were stained with X-Gal at 14.5 days of development, when the skin had not formed yet. Since we observed a perfect cartilage-specific pattern with four copies of the 48-bp segment linked to a 309-bp *Col2a1* promoter, it is likely that elements which inhibit expression in the brain, and possibly also in the skin, are located in the *Col2a1* promoter between −309 and −89 or in the 48-bp element outside the 18-bp enhancer. It is possible that the expression in brain and skin tissues corresponds to the low-level expression of Col2a1 revealed in these tissues by in situ hybridization (2). Although we have not tested the 89-bp *Col2a1* promoter by itself in transgenic mice, it is unlikely that the cartilage expression observed with the construction containing the 18-bp enhancer and the 89-bp *Col2a1* promoter was generated by the promoter itself since a 309-bp *Col2a1* promoter was unable by itself to confer cartilage expression in transgenic mice (28).

While the manuscript was being completed, a 100-bp segment of the rat *Col2a1* intron 1 was reported as having the minimum size necessary for chondrocyte-specific expression in DNA transfection experiments (13). This element extends 41 bp upstream and 12 bp downstream of the 48-bp enhancer segment described here. One substitution mutation and two internal deletions in a region corresponding to our 18-bp enhancer greatly decreased activity, whereas internal deletions in an AT-rich region in the 5′ portion of this 100-bp element reduced activity by about one-half. We hypothesize that the AT-rich sequence can potentiate the promoter activation generated by the 18-bp chondrocyte enhancer.

In conclusion, we have identified an 18-bp segment in the first intron of the mouse Col2a1 gene that was sufficient to direct promoter expression in chondrocytes of transgenic mice and in transiently transfected RCS cells and primary chondrocytes. Mutation in a 10-bp sequence within this segment or deletion of these 10 bp prevented enhancer activity and also abolished the binding of proteins specifically expressed in RCS cells and chondrocytes. These proteins likely belong to the POU domain protein family. We speculate that they are key factors in the activation of the Col2a1 gene in chondrocytes and might also be involved in the expression of other genes selectively activated during chondrocyte differentiation. Our data also pointed out other elements located in the first intron and in the promoter of the Col2a1 gene which likely bind proteins that help generate the high level of expression of the Col2a1 gene in chondrocytes.

ACKNOWLEDGMENTS

This work was funded by NIH grants AR40335 and AR42909 (to B.D.C.). V.L. was the recipient of a postdoctoral fellowship from the Arthritis Foundation. DNA sequencing was performed by The University of Texas M. D. Anderson Cancer Center Core Sequencing Facility, which is supported by NCI grant CA16672.

We are very grateful to Michael Perry for the generous gift of Oct-1 antibodies, to Lee Ann Garrett for helpful advice, to Sandra McKinney for expert technical assistance, and to Françoise Constry, Su Sin Chen, and Antonella Pellegrino for providing nuclear extracts from various cell types.

REFERENCES

1. Cancedda, R., F. Descalzi Cancedda, and P. Castagnola. 1995. Chondrocyte differentiation. Int. Rev. Cytol. 159:265–358.
2. Cheah, K. S., E. T. Lau, P. K. Au, and P. P. Tam. 1991. Expression of the mouse α1(II) collagen gene is not restricted to cartilage during development. Development 111:945–953.
3. Digman, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11:1475–1489.
4. Dyer, R. B., and N. K. Herzog. 1995. Isolation of intact nuclei for nuclear extract preparation from a fragile B-lymphocyte cell line. BioTechniques 19:192–195.
5. Erlebacher, A., E. H. Filvaroff, S. E. Gitelman, and R. Derynck. 1995. Toward a molecular understanding of skeletal development. Cell 80:371–378.
6. Friedrich, G., and P. Soriano. 1991. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. Genes Dev. 5:1513–1523.
7. Garofalo, S., E. Vuorio, M. Metsäranta, R. Rosati, D. Toman, J. Vaughan, G. Lozano, R. Mayne, J. Ellard, W. Horton, and B. de Crombrugghe. 1991. Reduced amount of cartilage collagen fibrils and growth plate anomalies in transgenic mice harboring a glycine-to-cysteine mutation in the mouse type II procollagen α1-chain gene. Proc. Natl. Acad. Sci. USA 88:9648–9652.
8. Goldberg, H., T. Helaakoski, L. A. Garrett, G. Karsenty, A. Pellegrino, G. Lozano, S. N. Maity, and B. de Crombrugghe. 1992. Tissue-specific expression of the mouse α2(I) collagen promoter: studies in transgenic mice and in tissue culture cells. J. Biol. Chem. 267:19622–19630.
9. Herr, W., and M. A. Cleary. 1995. The POU domain: versatility in transcriptional regulation by a flexible two-in-one DNA-binding domain. Genes Dev. 9:1679–1693.
10. Hinkley, C., and M. Perry. 1992. Histone H2B gene transcription during Xenopus early development requires functional cooperation between proteins bound to the CCAAT and octamer motifs. Mol. Cell. Biol. 12:4400–4411.
11. Horton, W., T. Miyashita, K. Kohno, J. R. Hassell, and Y. Yamada. 1987.

All for one and one for all: condensations and the initiation of skeletal development

Brian K. Hall* and T. Miyake

Summary

Condensation is the pivotal stage in the development of skeletal and other mesenchymal tissues. It occurs when a previously dispersed population of cells gathers together to differentiate into a single cell/tissue type such as cartilage, bone, muscle, tendon, kidney, and lung and is the earliest stage during organ formation when tissue-specific genes are upregulated. We present a synopsis of our current understanding of how condensations are initiated and grown, how their boundaries and sizes are set, how condensation ceases, and how overt differentiation begins. Extracellular matrix molecules, cell surface receptors and cell adhesion molecules, such as fibronectin, tenascin, syndecan, and N-CAM, initiate condensation formation and set condensation boundaries. *Hox* genes (*Hoxd-11-13*) and other transcription factors (CFKH-1, MFH-1, osf-2), modulate the proliferation of cells within condensations. Cell adhesion is ensured indirectly through *Hox* genes (*Hoxa-2*, *Hoxd-13*), and directly via cell adhesion molecules (N-CAM and N-cadherin). Subsequent growth of condensations is regulated by BMPs, which activate *Pax-2*, *Hoxa-2* and *Hoxd-11* among other genes. Growth of a condensation ceases when Noggin inhibits BMP signalling, setting the stage for transition to the next stage of skeletal development, namely overt cell differentiation. *BioEssays* 22:138–147, 2000. © 2000 John Wiley & Sons, Inc.

Introduction

Cell condensation is the third of four primary phases of skeletogenesis. The first is the migration of cells to the site of future skeletogenesis; the second is the tissue (epithelial-mesenchymal) interactions that result in condensations forming; and the fourth is the overt differentiation of chondroblasts or osteoblasts (Fig. 1). Each phase involves different cellular processes and separate genetic controls.

Our focus in this article is on the process of condensation. It is the pivotal stage in skeletal development and takes place when a previously dispersed population of mesenchymal cells forms an aggregation or condensation, which is the earliest sign of the initiation of a skeletal element or elements.[1,2] We say elements, because patterning within condensations can be quite complex, with more than one bone or cartilage arising from a single condensation. For example:

- two cartilages of the lower jaw and two of the bones of the middle ear (the incus and the malleus) in mouse embryos arise from a single condensation (Fig. 2);[2,3]
- the seven bones of the lower beak of chick embryos arise from a single condensation;[4]
- three bones of the zebrafish head arise from a single condensation.[5]

Condensations can also be complex temporally; the subunits of a condensation may persist for different lengths of time before making the transition to overt cell differentiation (Fig. 2).[2,3,6,7]

Skeletal condensations were first described—and the term condensation first applied to them—in a now classic study on the development of long bones of embryonic chicks published by a pioneer in tissue culture, Honor (later Dame Honor) Fell in 1925;[8] see Reference 9 for a review of the early work. The mammalian geneticist Hans Grüneberg found that so many mutations affecting skeletal development acted at the condensation state that he named condensations the "membranous skeleton" that precedes the cartilaginous and/or osseous skeletons.[10] Condensations are the primary resource from which the skeleton is built and through which the skeleton is modified ontogenetically and phylogenetically. Atchley and Hall[11] identified condensations as the fundamental cellular units of morphological change in organogenesis during vertebrate evolution; also see References 12–15.

Condensations may be recognised either at the cellular level—through the closer packing density that characterises condensed from uncondensed cells—or at the molecular level. As shown in Figure 3, prechondrogenic condensations have cell surface molecules that bind peanut agglutinin lectin and allow condensations to be visualised;[3,16,17] the affinity of prechondrogenic cells in condensations for peanut agglutinin (PNA) is utilised in a technique to isolate and characterise PNA-positive cells.[18] Condensations also have elevated levels of such extracellular matrix or cell surface molecules as hyaladherins, versican, tenascin, syndecan, N-CAM, and heparan sulphate and chondroitin sulphate

Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J1. E-mail: BKH@IS.DAL.CA
*Correspondence to Brian K. Hall, Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J1.
Funding agency: The Natural Sciences and Engineering Research Council of Canada; Grant number: A5056.

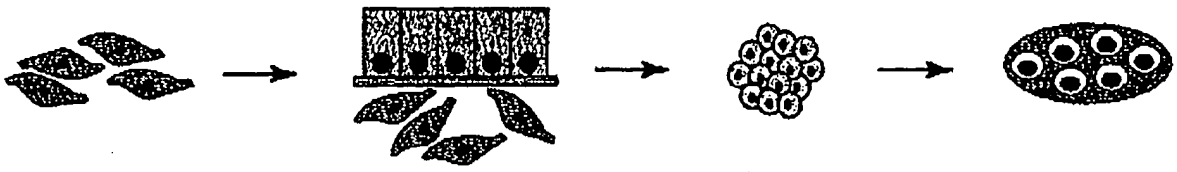


Figure 1. The four phases of the development of a skeletal element are (from left to right): migration of preskeletal cells (green) to the site of future skeletogenesis, which is always associated with an epithelium (purple) and epithelial basement membrane (brown); interactions of those cells with epithelial cell products resulting in initiation of a condensation (yellow); and overt differentiation of chondroblasts or osteoblasts (blue).

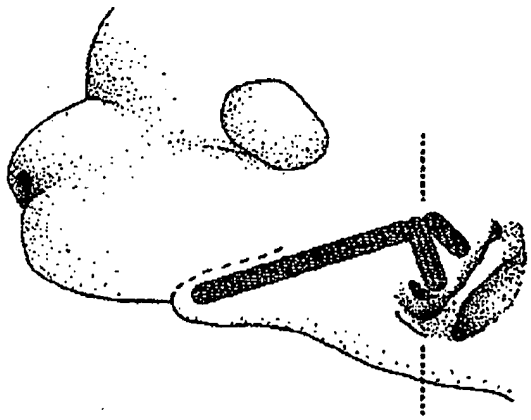


Figure 2. A diagrammatic representation of the components of the first arch prechondrogenic condensation in a C57BL/6 mouse embryo to demonstrate that several skeletal elements can arise from a single condensation and persist for varying periods of time. Components are coded as follows: Blue, the core component that gives rise to Meckel's cartilage, and which is present for 12 hours; brown, a rostral component from which arises the symphyseal cartilage between the two lower jaws and which persists for 28 hours; green, a caudal component that lies across the mandibular-hyoid arch boundary (dotted line), persists for 30 hours and from which the malleus arises; red, the most caudal component, which also has a duration of 30 hours, and from which the incus arises.

proteoglycans. The discovery of other condensation markers continues, however. Thrombospondin-4, one of five related glycoproteins, is expressed transiently in mesenchyme associated with both chondrogenic and osteogenic condensations.[19] The sequence of events in condensation is described in the sections that follow. We begin with the events that initiate condensation.

Initiation of condensations

Condensations form as a result of one or a combination of three processes:

- enhanced mitotic activity;
- aggregation of cells toward a center; and
- failure of cells to disperse from a center.[1,2]

By 1992, it was evident that TGF-β and fibronectin were involved in condensation formation.[1] TGF-β regulates fibronectin, which in turn regulates the cell adhesion molecule, N-CAM (Fig. 3, Table 1). By 1995, we and others could model condensation as resulting from epithelial-mesenchymal interactions controlled by TGF-β, BMP-2, *Msx-1*, and tenascin.[2,20-22] BMP receptors have now been characterised and localised to condensing mesenchyme in chick limbs and in condensations in murine embryos.[23]

Levels of intracellular cAMP increase when prechondrogenic cells condense. The increase is associated with phosphorylation of p35, the nuclear substrate for cAMP-dependent protein kinase (PKA). Indeed, within the chondrogenic pathway, PKA is found only in the nuclei of condensing cells and not in chondrocyte nuclei; PKA is imported into the nucleus at condensation.[24] The cell-to-cell interactions concomitant with condensation and which elevate cAMP levels may in turn mediate upregulation of chondrogenic genes, thereby providing a mechanism for initiating condensation and skeletal development.

Pax-1 and *Pax-9*, genes that share the paired-type DNA-binding homeodomain and encode nuclear transcription factors, have their strongest expression at the condensation stage, consistent with a role in condensation (Fig. 3).[25] *Pax* genes are important regulators of epithelial-mesenchymal interactions in many tissues and organs including the skeleton, kidneys, sense organs (eyes, ears, nose), limb muscle, and brain. *Pax-2*, which is regulated by BMP-7, is an important player regulating condensation size (Fig. 3).[26]

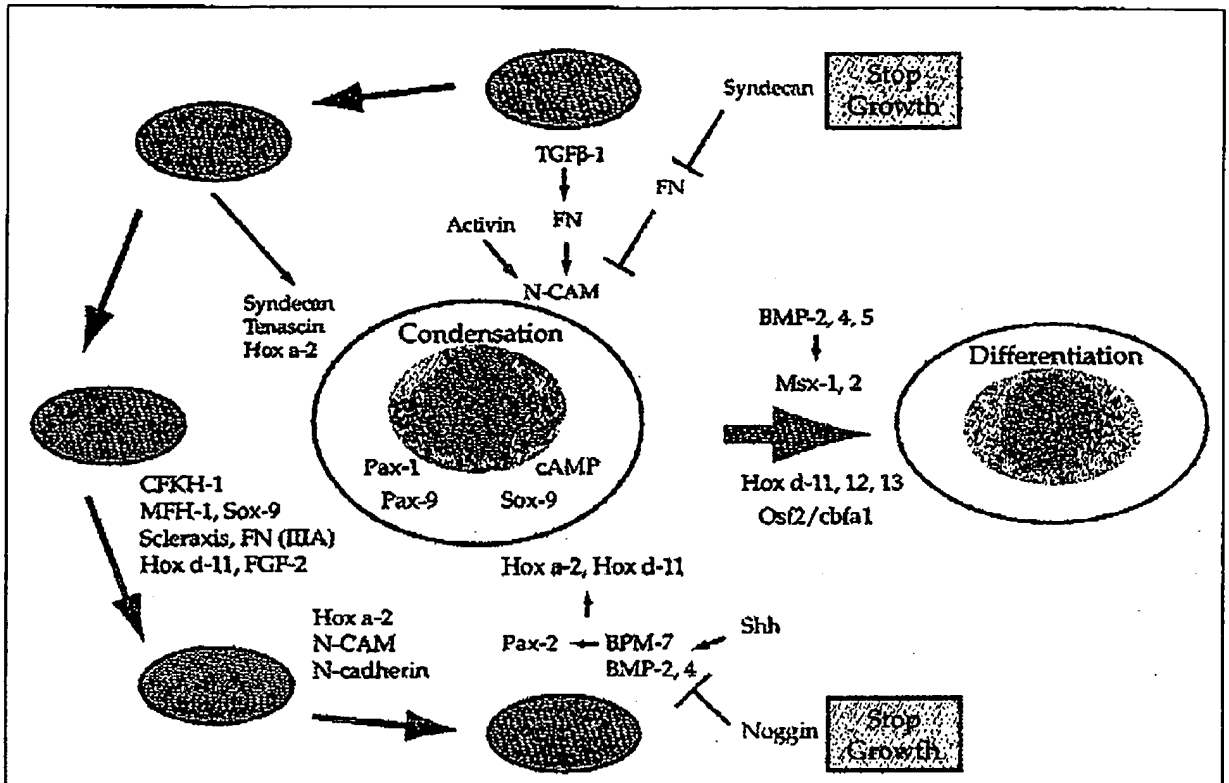


Figure 3. A summary of the major genes and gene products involved in condensation formation (blue) and in the transition from condensation to overt differentiation (green arrow). The condensation is visualised using peanut agglutinin lectin: the elevated levels of cyclic-AMP and major genes expressed at the condensation stage (*Pax-1, Pax-9, Sox-9*) are shown. The differentiating cartilage is visualised with Alcian blue. The major genes associated with five stages in condensation (Initiate; Set Boundary; Proliferate; Adhere, Grow) are shown. Two pathways that stop condensation growth (Stop Growth) are shown in yellow and red. Cessation of condensation leads to differentiation (green arrow), which involves both upregulation of genes to initiate differentiation and downregulation of genes to terminate condensation. See text for details.

Epithelial-mesenchymal interactions in avian skeletogenesis are mediated, in part, by *Prx-1* (formerly *MHox*) and *Prx-2*, two members of a subclass of the paired-class of homeobox genes related to *Drosophila aristaless*. Both genes also play a role in mediating interactions between perichondrial cells and adjacent chondrocytes,[27] providing a molecular link between condensation and perichondrial development. The presence of *Hox*-gene-binding sites in the upstream regulatory element of the N-CAM promoter makes N-CAM a potential downstream target for *Hox* genes.[28] *Prx-1* is another potential upstream regulator of CAMs and therefore of condensation initiation.[29,30]

In murine embryos, *Alx-3*—a mouse paired (*aristaless*)-like homeobox gene—is expressed at the condensation stage in both ectomesenchyme and lateral plate mesoderm. Expression is in domains that overlap with *Prx-2* and with *Cart-1*, a chondrogenic marker.[31] *Alx-4* is found in murine mesenchymal condensations of the skull, hair, teeth, and mammary glands. Null mutants have only preaxial polydactyly,[32] indicating either that *Alx-4* plays a minor role in skeletogenesis or that its function(s) overlap with other genes.

FGFs may have a greater role in condensation than has been appreciated so far. Homologues of human FGF receptors -1, -3, and -4 are found in high levels in cranial, branchial arch, limb, and axial skeletogenic mesenchyme in the European salamander *Pleurodeles waltl*.[33] Such high levels are indicative of a role for FGFs in condensation initiation or maintenance.

TABLE 1. The Major Classes of Genes and Gene Products Associated with Skeletogenic Condensations Along with Their Functions and Stages of Action[a]

Gene/gene product	Function	Stage
	Growth factors	
BMPs	regulate Hox genes (*Hoxa-2*, *Hoxd-11*, *Pax-2*) in response to *Shh*	growth
	regulate *Msx-1*, *Msx-2*	transition to differentiation
FGF-2	regulates N-CAM	initiation, proliferation, growth
TGF-β	regulates fibronectin	initiation
	Cell surface, cell adhesion and Extracellular matrix molecules	
Fibronectin	an extracellular glycoprotein regulated by TGF-β; regulates N-CAM	initiation, proliferation
N-cadherin	a cell adhesion molecule	adhesion
N-CAM	a cell adhesion molecule regulated by FN, *Prx-1*, *Prx-2* and FGF	initiation, adhesion
Noggin	a secreted protein that binds to and inactivates *BMP-2*, *BMP-4*, *BMP-7*	slows or stops growth
Syndecan	a receptor that binds to tenascin; binds to fibronectin to inactivate N-CAM	sets boundary
Tenascin	an extracellular glycoprotein that binds to syndecan	stops condensation growth sets boundary
	***Hox* genes**	
Hoxa-2	regulated by BMPs, downregulated *cbfa-1*	sets boundary, growth, prevents differentiation
Hoxa-13	alters adhesive properties	adhesion
Hoxd-11	regulated by BMP	proliferation, growth
Hoxd-11-13	transcriptional activation	transition to differentiation
	Transcription factors	
cbfa-1	transcriptional activating protein inhibited by *Hoxa-2*	differentiation of chondroblasts
CFKH-1	a chicken forkhead-Helix transcription factor that regulated TGF-β and interacts with *Smad* transcription factors	initiation, proliferation
MFH-1	mesenchymal transcription factor	proliferation
osf-2	transcriptional activation protein regulated by BMP-7 and Vitamin D_3	switches cells into the osteoblastic pathway
Pax-1, *Pax-9*	encode nuclear transcription factors, regulated by BMP-7	growth
Prx-1, *Prx-2*	upstream regulation of N-CAM	initiation
Scleraxis	a basic helix-loop-helix protein	proliferation
Sox-9	regulates the collagen 2α1 gene	proliferation

[a]Also see Figure 3.

Establishing the boundaries of condensations

The association between TGF-β and tenascin in epithelial-mesenchymal interactions documented in Hall and Miyake[2] has been further confirmed experimentally using mouse limb mesenchyme in culture. TGF-β-1 upregulates a number of molecules associated with prechondrogenic condensations, including tenascin, fibronectin, N-CAM, and N-cadherin.[34] N-cadherin is associated with adhesion of cells in condensations, N-CAM with stabilisation or maintenance of condensations (Fig. 3). Surprisingly, elimination of N-CAM does not affect condensation initiation, but this may reflect a degree of functional redundancy with other molecules that mediate adhesion.[35]

Our understanding of the roles of tenascin and syndecan has been enhanced through the demonstration that early condensations for limb cartilages in chick embryos are surrounded by a cell layer that is strongly syndecan-3-positive.[36] Syndecan plays an important role in establishing the boundary conditions that set the limits to condensation size (Fig. 3). Syndecan-3 has also been demonstrated in other areas of epithelial-mesenchymal interactions association with formation of condensations, including those involved in the development of the lens, the otic placode, sclerotomes, and feathers.[37] Syndecan-3 is also found in areas outside the developing skeleton where basic patterning occurs, namely in the floor plate of the ventral neural tube and in the distal limb mesenchyme.[37] The possibility that syndecan-3 may play its patterning role through action on cell-cell and cell-matrix interactions is an intriguing one worthy of further study.

FGFs are another class of molecules that mediate condensation in various organ systems, often acting at more

than one stage in a cascade of interactions.[38] BMP and FGF are involved in another situation where skeletogenic cells are maintained at a boundary with non-skeletogenic cells, viz. in developing sutures. Expansion of the osteogenic front is associated with intense expression of BMP-2 and BMP-4 and upregulation of *Msx-1* and *Msx-2*. *Msx-1* is also found in growing facial mesenchyme in which condensations will form.[39] Closure of sutures is speeded up when beads soaked in FGF-4 are implanted into the suture front. Therefore, as in condensation, maintenance of the population of preosteogenic cells at suture margins is mediated by signals involving FGF, BMP, and Msx.[40]

Syndecan and tenascin also help to set boundary conditions in perichondria and periostea.[41] Thus, tenascin and syndecan are deployed in those situations where a chondrogenic or osteogenic population must be set off from surrounding non-skeletogenic mesenchyme. Tenascin-C is also associated with the ectomesenchymal condensations of the mandibular skeleton in chick embryos, both the prechondrogenic condensation for Meckel's cartilage and the preosteogenic condensations for the membrane bones of the mandible.[42] Thus, we see a role for tenascin-C and syndecan-3 in ectomesenchymal (neural-crest-derived) skeletogenic condensations as in condensations derived from mesoderm (axial and appendicular skeleton).

Although involved in condensation, expression of tenascin may not be essential for condensation or for skeletal development, both of which are normal in mice after the single copy of the tenascin gene is knocked-out.[43] I say "may not be essential," because at this time we cannot rule out the possibility that another gene(s) compensates for the absence of tenascin. Because of partial functional redundancy and/or overlapping gene functions, the interpretation of knock-out experiments is not as clear as one might hope and as originally anticipated.

Adhere, proliferate, and grow

Transcription factors that play pivotal roles in condensation formation or in the transition from condensation to overt differentiation have been identified over the past several years.

Cfkh-1

A chicken forkhead (winged)-Helix transcription factor expressed in condensations of both mesodermal (ribs, vertebrae, limbs) and neural crest (branchial arch) origin, is downregulated as chondrocytes or osteoblasts differentiate.[44] The most likely function of CFKH-1 is promotion of proliferation at the condensation stage (Fig. 3, Table 1) by mediating the effects of TGF-β on transcription of its target genes, such as fibronectin by interacting with Smad transcription factors.[45]

Mesenchyme fork head 1 (mfh-1)

Mfh-1 is transcription factor expressed in sclerotomal and craniofacial mesenchyme and in differentiating cartilage of murine embryos. MFH-1-deficient mice display skeletal defects consistent with a possible role for MFH-1 in proliferation of condensations.[46] A further transcription factor, *Sox-9*, is expressed in murine chondrogenic condensations, where it regulates the col2α1 gene. As with MFH-1, deficiencies in *Sox-9* lead to skeletal defects.[47] Scleraxis, a basic helix-loop-helix protein expressed at maximal levels in axial, appendicular, and craniofacial chondrogenic condensations, is downregulated with chondrogenic differentiation.[48] Clearly, further research will be required to understand the relative roles of these various transcription factors.

Hoxa-2 expression is specifically excluded from chondrogenic condensations in the second branchial arch of murine embryos, a pattern that is consistent with *Hoxa-2* playing a role in establishing the boundary and/or size of the condensation.[49] *Hoxa-13*, on the other hand, alters the adhesive properties of prechondrogenic cells. Therefore, *Hox* genes have to be considered as modulators of cell adhesion (Fig. 3). Infection of limb mesenchyme with a *Hoxa-13* construct and subsequent culture of these cells is followed by aggregation of the *Hoxa-13*-expressing cells into multiple clusters.[50,51] Because limb mesenchyme contains cells from both somitic and lateral plate mesoderm that give rise to myogenic, chondrogenic, and fibroblastic cell lineages, similar experiments need to be undertaken using cells from individual condensations.

Position and shape

Homeobox genes are now known to play greater roles in both epithelial-mesenchymal interactions and in condensation formation than previously suspected. Indeed, *Hox* genes are involved in determination of such fundamental attributes of condensations as timing and position and can affect the particular shapes that condensations assume.

5' *Hoxd* genes (*Hoxd 9-13*), which are known to be involved in skeletal patterning, have now been shown to function at the mesenchymal condensation stage of skeletogenesis. Their patterns of expression in chick limb mesenchyme maintained in vitro are complex; *Hoxd-9* and *Hoxd-13* exhibit one pattern, *Hoxd-10-12* another. Early in culture, *Hoxd-9* and *-13* are expressed only in some cells, while *Hoxd-10*, *d-11*, and *d-12* are expressed in all cells. As chondrogenesis ensues, *Hoxd-9* and *d-13* are only expressed in chondrogenic cells, *Hoxd-10-d12* in both chondrogenic and nonchondrogenic cells.[52] Furthermore, at least in chick limb buds, cartilage can function as a signalling center, inducing

expression of *Hoxd-12*, *Hoxd-13*, and *Shh*, which are in turn associated with induction of supernumerary cartilages.[53]

Misexpression of *Hoxd-13* and *Hoxd-11* in chick limb buds results in skeletal defects that can be traced to two stages of chondrogenesis: *Hoxd-11* acts at both the condensation stage and during later chondrogenesis; *Hoxd-13* acts only at the later stage (Fig. 3).[54]

Double knock-out of *Hoxa-11* and *Hoxd-11* results in skeletal defects traceable to the condensation stage and illustrates functional cooperation between paralogous *Hox* genes.[55] There is also cooperation between non-paralogous *Hox* genes; *Hoxd-11* and *Hoxa-10* exhibit functional cooperation, as judged from the observation that double mutants have more extreme phenotypes that single mutants.[56] Differential expression of *Hoxa-11* in *Xenopus* fore- and hindlimbs suggests that some gene expression patterns at the condensation stage pattern the proximal tarsal elements in *Xenopus* in a way not mirrored in the upper limb.[57]

Establishing condensation size

Individual condensations have predictable sizes. The size of a condensation and therefore, by inference, the number of cells within it, affects whether skeletogenesis will be initiated. Reduce a condensation below a critical threshold and skeletogenesis may not begin. Increase the size of a condensation and an overly large skeletal element can form.

This statement is supported by studies on mutant embryos in which missing, small, or abnormally shaped skeletal elements can be traced to deficiencies in the membranous skeleton.[1,2] *Talpid*^{3} chick embryos and *Brachypod (bp*^{J}*)*, *Congenital Hydrocephalus (ch)*, *Short Ear*, and *Phocomelia (Pc)* mice are well characterised mutations that act initially at the condensation stage but that act through different genetic pathways. *Talpid*^{3} reflects overexpression of N-CAM; *Brachypod* is a frameshift mutation in Growth and Differentiation factor 5 (GDF-5), while *Short Ear* is a mutation in BMP-5. The number of cells in a condensation is therefore a realistic reflection of the growth potential of individual skeletal elements, as Grüneberg[10] concluded 35 years ago.

The initial action of mutations at the condensation stage underscores condensation as the first major stage of selective gene activation in skeletogenesis, or indeed in odontogenesis, myogenesis, or ligamentogenesis. Genes specific to the differentiation pathway are upregulated at condensation; genes not specific to the pathway are not.

BMPs

Condensation size is regulated through signalling pathways involving BMP-2 and BMP-4 (Fig. 3, Table 1).[7] Overexpression of both of these growth factors in chick embryos is followed by dramatic increases in both size and shape of skeletal elements. Enhanced recruitment of mesenchymal precursors to cartilage condensations—and to perichondria, which contribute cells to anlage in vivo or to nodules in vitro in the same way that condensations grow by accretion[58]—are likely mechanisms of the BMP effect; BMP-2 is expressed in mesenchyme surrounding condensations and so is critically located to modulate expansion of condensation size.[59] Recruitment of cells to condensations by BMP is also consistent with the known action of BMP in inducing ectopic bone in adults: implanting BMP outside the skeleton elicits a cascade of events—condensation, overt differentiation of cartilage, vascular invasion, and replacement of cartilage by bone.[60,61] Calcifying vascular cells obtained from aortic cell cultures also condense before forming nodules that mineralise.[62] This observation reinforces the idea that condensation is an essential initial step in ectopic mineralisation, as it is in normal and ectopic skeletogenesis.

Fibronectin

Fibronectin is known to be critical for early embryogenesis. Homozygote mutant embryos lacking fibronectin implant and begin gastrulation but fail to form notochords or somites and have deformed hearts and circulation.[63]

An alternatively spliced exon (IIIa), one of the functional portions of fibronectin involved in condensation, is downregulated immediately after the condensation phase during limb chondrogenesis.[64] Exposing micromass cultures of limb mesenchyme to an antibody against the exon disrupts condensation and inhibits subsequent chondrogenesis. Similarly, injection of an antibody into limb buds in ovo results in embryos with smaller limbs and fewer limb elements.

Surprisingly, levels of fibronectin and fibronectin mRNA are higher in prechondrogenic condensations from chick leg buds than in wing bud condensations. Condensations that develop when mesenchyme from the two limb types is maintained in vitro differ in morphology and in distribution of fibronectin. Wing condensations are broad and flat with much diffusely organised fibronectin; leg condensations are compact and spherical and connected by fibronectin-rich fibers. Wing bud condensations are more sensitive to TGF-β, which increases fibronectin levels and in turn increases condensation size. Treating cultured wing bud mesenchyme with an antibody against the amino-terminal heparin-binding domain of fibronectin inhibits condensation formation. Similar treatment of leg bud mesenchyme has no effect on condensation development.[65] Comparative analysis of wing and leg bud condensations is therefore a promising system in which to analyse the basis for differences between condensations and how condensation size is set.

Extrinsic control

As well as being set by such intrinsic mechanisms as recruitment of BMP-2-positive cells to a condensation, the size of a condensation can also be set extrinsically. The

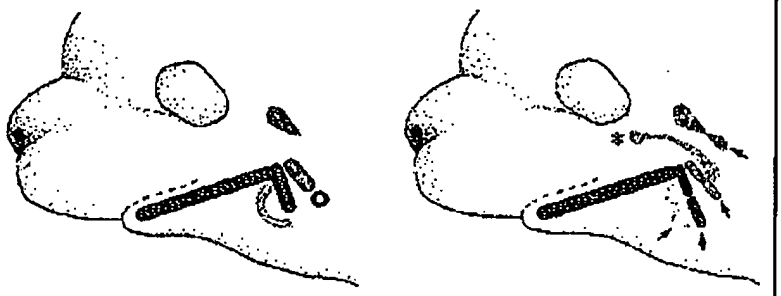


Figure 4. The lower jaw and middle ear skeletal elements of a wild-type mouse (left) are compared with those found in a mouse in which the gene *Hoxa-2* was knocked-out by Rijli et al.[69] The condensations from which these elements form are shown in Figure 1. Elements that are duplicated in the knock-out mouse are marked with an arrow. The * identifies an ectopic cartilage (gray), which only exists as a condensation in the wild-type (not shown). Meckel's cartilage (blue) is unaffected by the loss of *Hoxa-2*. The middle ear ossicles—tympanum (yellow), malleus (green), and incus (orange)—are all duplicated, as is the squamosal (pink). The stapes (black) is missing, while an ectopic cartilage (gray, *) lies between the skull and the duplicated incus.

condensations for avian dorsal root ganglia (DRG) in brachial segments 14 and 15 are more than 80% larger than the condensations for DRG in cervical segments 5 and 6. The increased cell number in brachial DRG is not because more neural crest cells colonise brachial ganglia, that is, it is not intrinsic to the condensations. Rather, it reflects specific influences from the sclerotomal mesoderm in the regions in which the DRG develop.[66] How such extrinsic influences as mechanical and endocrine control, which are so important to subsequent skeletal development, influence the size of skeletal condensations, is worthy of further study.

Condensation size and initiation of differentiation

Recent studies affirm the importance of a critical condensation size if cells are to progress from condensation to initiation of chondrogenic or osteogenic differentiation.[67] Mice in which *Hoxa-2*, *Dlx-2*, *MHox*, *Otx*, or the retinoic acid receptor have been knocked-out display ectopic or apparently duplicated skeletal elements. Two examples will be used to illustrate this approach. Both involve targeted deletion of *Hoxa-2*.

Gendron-Maguire et al.[68] deleted exon 1, the first 32 base pairs of exon 2, the splice acceptor site, and all introns from *Hoxa-2*. Rijli et al.[69] deleted exon 1, the first 72 base pairs of exon 2, and the translation initiation site. In both homozygous knock-outs, there was abnormal development of the malleus and incus of the middle ear and what appeared to be mirror-image duplications of the malleus and incus and of the tympanic bone of the skull. An ectopic squamosal bone also formed. Stapes and stylohyal cartilages were missing in both sets of embryo but an ectopic cartilage formed (Fig. 4).

In a thoughtful comparative analysis of the skeletal phenotypes of such mice, Smith and Schneider[70] detected a common underlying theme. An excess of mesenchymal cells accumulates at sites that normally possess very small condensations that do not differentiate. Because of the accumulation of additional mesenchyme at these sites, chondrogenesis is initiated, resulting in the formation of ectopic cartilages as shown in Figure 4. The site of this condensation—between the murine alisphenoid and incus (epipterygoid and quadrate in reptiles)—and its differentiation in knock-out mice, allows a cartilage to form that bears a superficial resemblance to the reptilian palatoquadrate. Indeed, ectopic "quadrate-like" and "pterygoquadrate-like" bones in *Hoxa-2* knock-out mice were interpreted as atavistic reversals to a reptilian condition.[69] Smith and Schneider[70] however, dispute this, seeing no need to invoke atavistic interpretations when a more mechanistic explanation is sufficient—the differentiation of cartilage or bone in centres of condensation that are normally too small to initiate skeletogenesis. (See Newman and Tomasek[15] and Hall[21] for discussions of physical/proximate and evolutionary/genetic mechanisms in skeletal evolution.) While "neomorphic" in the sense that the cartilages do not normally form, the elements are not atavistic since the rudiment is normally present in all individuals, although small and unexpressed.

Miyake et al.[5] had previously emphasised the significance of the origin of these skeletal elements from neural crest cells from more than one region of the hindbrain, and argued that mesenchyme from both mandibular and hyoid arches contributes to the skeletal condensations of the auditory region. Smith and Schneider[70] also propose, however, that disruption of normal neural crest cell migration is the most likely mechanism responsible for increased condensation size. Their cautionary emphasis—that genes supply products for differentiative and morphogenetic pathways rather than specifying phenotypes—is an important one. In

order to elucidate the effects on the development of condensations of gene knock-out experiments, developmental series of embryos must be evaluated, since analysis of the phenotype at a single end point is insufficient to establish underlying mechanisms.

From condensation to overt differentiation

Progressing from condensation to overt differentiation of cells identifiable as chondroblasts (Fig. 3) or osteoblasts requires down-regulation of both N-CAM and the genes controlling proliferation and up-regulation of genes associated with differentiation. Thus, progression to the differentiation phase is achieved through signals that stop condensation growth, and so favour differentiation indirectly (with syndecan binding to fibronectin down-regulating N-CAM), and through signals that initiate differentiation directly, through such pathways as BMP-2, BMP-4, BMP-5, and the activation of homeobox genes such as *Msx-1* and *Msx-2* (Fig. 3, Table 1). A subsequent transition from proliferating chondroblasts to chondrocytes and hypertrophic chondrocytes is regulated via Indian hedgehog (Ihh), which induces PTH and PTH-related peptide receptor. PTHrP in turn acts via negative feedback to regulate Ihh and so controls the size of developing growth plates.[71,72] The discrete stages during skeletogenesis are each controlled by specific cascades of factors.

BMP-2 and BMP-5, known to be expressed in condensations in vivo, are expressed in the chondrogenic cells line C3H/10T 1/2 in vitro.[23,73] Not all cell lines are similar, however. Exposure to BMP-2 allows Atdc5 cells to progress to chondroblast differentiation, bypassing a condensation phase.[74]

BMP favours recruitment of cells into condensations, a recruitment that is antagonised by the secreted protein Noggin. BMP-Noggin feedback is, therefore, an important means of regulating condensation location, size, and duration (Fig. 3, Table 1). Noggin has also been demonstrated in condensations and immature chondroblasts in both the limb and the mammalian skull and, in parallel fashion, to interact antagonistically with BMPs during limb development.[75,76] Condensations can form in the absence of Noggin, but they rapidly become hyperplastic. Subsequently, although cartilage maturation is normal, joints between adjacent skeletal elements fail to form.

BMPs do not induce fibronectin and act primarily *after* condensation initiation. In contrast, TGF-β regulates fibronectin and both TGF-β and fibronectin are involved in condensation *initiation*.[77,78]

Tenascin and its cell surface receptor syndecan play important roles in epithelial- mesenchymal interactions leading to condensation. Tenascin is up-regulated during this phase and also plays a role in the transition from condensation to overt differentiation. Binding of syndecan to fibronectin blocks N-CAM in the late condensation stage (Fig. 3). Consequently, further condensation growth is blocked and differentiation is facilitated.[2] N-CAM, which plays an important role in mediating cell-to-cell adhesion regulating condensation formation (N-CAM contains type-I collagen and heparin sulfate proteoglycan binding domains), is expressed in periostea in association with osteogenesis but is down-regulated when periosteal cells switch to chondrogenesis, as occurs when secondary cartilage differentiates in membrane bone periostea.[79,80] Curiously, healthy, fertile mice are born after knocking-out the N-CAM gene.[81] Given all that we know about N-CAM's role in condensation formation and the need to block N-CAM to halt condensation growth, other factors must normally act with N-CAM, as noted earlier.

The transcriptional activator cbfa-1 (core-binding factor-1, also known as osteoblast specific *cis*-acting element-2 [Osf2]), a protein that binds to the osteocalcin promoter, is expressed in prechondrogenic and preosteogenic condensations but then strictly in the osteogenic cell lineage, being downregulated in chondrogenic lineages.[82,83] The central function of cbfa-1 in osteoblast differentiation was confirmed by the demonstration that osteogenic genes are expressed in non-osteogenic cells in which cbfa-1 has been expressed. Regulated by BMP-7 and vitamin D_3 *cbfa-1* is a strong candidate for a switching gene that directs cells into the osteogenic pathway. Cbfa-1 is inhibited by *Hoxa-2* expression. In *Hoxa-2*- embryos, Cbfa-1 is expressed in the second arch. Thus *Hoxa-2* normally inhibits osteogenesis by down-regulating cbfa-1.[49] The key role of Cbfa-1 is shown by the fact that targeted disruption of cbfa-1 in mice completely blocks bone formation.[84] Thus, a variety of pathways are used as cells make the transition from condensation to overt differentiation.

Conclusions

Development of the skeleton is a stepwise set of processes, each step depending on the step before, but each involving different cellular processes (migration, adhesion, proliferation, growth) and each is subject to different genetic control. Condensation is the aggregation of cells that facilitates selective regulation of genes specific for either chondro- or osteogenesis. Condensations must attain a critical size and cells must interact within a condensation for the condensation phase to cease and differentiation to be initiated; it really is "all for one and one for all."

Condensation is itself a multistep process, involving initiation, establishment of boundary conditions, cell adhesion, proliferation, growth, and cessation of growth. The major genes in each phase are now known (Fig. 3, Table 1). Mutations affecting each phase are also known. The availability of natural mutations along with gene knock-out experiments provide powerful tools with which to analyse this pivotal

stage in skeletogenesis. Challenges for the future are to fully understand the cascades of genes regulating each phase of condensation formation, to identify functional redundancy between genes acting at different phases, to understand how condensation ceases and the differentiation phase begins, and to place ourselves in a position to manipulate each phase in order to correct skeletal defects that have their origin at the condensation stage.

Acknowledgments

Gratefully acknowledged are the very helpful comments from two anonymous referees. This article is dedicated to the memory of Peter Thorogood, a pioneer in the study of condensations.

References

1. Hall BK, Miyake T. The membranous skeleton: the role of cell condensations in vertebrate skeletogenesis. Anat Embryol 1992;186:107–124.
2. Hall BK, Miyake T. Divide, accumulate, differentiate: cell condensation in skeletal development revisited. Int J Dev Biol 1995;39:881–893.
3. Miyake T, Cameron AC, Hall BK. Stage-specific onset of condensation and matrix deposition for Meckel's and other first arch cartilages in inbred C57BL/6 mice. J Craniofac Genet Dev Biol 1996;16:32–47.
4. Dunlop L-LT, Hall BK. Relationships between cellular condensation, preosteoblast formation and epithelial-mesenchymal interactions in initiation of osteogenesis. Int J Dev Biol 1995;39:357–371.
5. Schilling TF, Kimmel CB. Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo. Development 1997;124:2945–2960.
6. Hall BK, Miyake T. How do embryos measure time? In: McNamara KJ, editor. Evolutionary change and heterochrony. New York: John Wiley & Sons; 1995. p 3–20.
7. Miyake T, Cameron AC, Hall BK. Variability of embryonic development among three inbred strains of mice. Growth Dev Aging 1997;61:141–155.
8. Fell HB. The histogenesis of cartilage and bone in the long bones of the embryonic fowl. J Morph Physiol 1925;40:417–459.
9. Hall BK. Introduction: research in the development and structure of the skeleton since the publication of Murray's Bones. In: Murray PDF, editor. Bones: a study of the development and structure of the vertebrate skeleton, with an introduction by DK Hall. Cambridge: Cambridge University Press; 1985 p xi–xix.
10. Grüneberg H The pathology of development. a study of inherited skeletal disorders in animals. Oxford: Blackwells Scientific Publications. 1963.
11. Atchley WR, Hall BK. A model for development and evolution of complex morphological structures. Biol Rev 1991;66:101–157.
12. Smith MM, Hall BK. Evolutionary and developmental origins of vertebrate skeletogenic and odontogenic tissues. Biol Rev 1990;65:277–374.
13. Smith MM, Hall BK. A developmental model for evolution of the vertebrate exoskeleton and teeth: the role of cranial and trunk neural crest. Evol Biol 1993;27:387–448.
14. Ettinger L, Doljanski F. On the generation of form by the continuous interactions between cells and their extracellular matrix. Biol Rev 1992;67:459–489.
15. Newman SA, Tomasek JJ. Morphogenesis of connective tissues. In Comper WD, editor. Extracellular matrix, Volume 2. Molecular components and interactions. Amsterdam: Overseas Publishers Association; 1996. p 335–369.
16. Zschäbitz A, Krahn V, Gabius HJ, Weisor H, Khaw A, Biesalski HK, Stofft E. Glycoconjugate expression of chondrocytes and perichondrium during hyaline cartilage development in the rat. J Anat 1995;187:57–63.
17. Zschäbitz A. Glycoconjugate expression and cartilage development of the cranial skeleton. Acta Anat 1998;61:254–274.
18. Stringa E, Tuan RS. Chondrogenic cell subpopulation of chick embryonic calvarium: isolation by peanut agglutinin affinity chromatography and in vitro characterization. Anat Embryol 1996;194:427–437.
19. Tucker RP, Adams JC, Lawler J. Thrombospondin-4 is expressed by early osteogenic tissues in the chick embryo. Dev Dyn 1995;203:477–490.
20. Raff M. Size control: the regulation of cell numbers in animal development. Cell 1996;86:173–175.
21. Hall BK. Evolutionary developmental biology. 2nd edition. Dordrecht, Netherlands: Kluwer Academic Publishers. 1998.
22. Peters H, Balling R. Teeth: where and how to make them. Trends Genet 1999;15:59–65.
23. Zou H, Wieser R, Massagué J, Niswander L. Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. Genes Dev 1997;1:2191–2203.
24. Zhang Q, Carr DW, Lerr KM, Scott JD, Newman SA. Nuclear localization of type II cAMP-dependent protein kinase during limb cartilage differentiation is associated with a novel developmentally regulated A-kinase anchoring protein. Dev Biol 1996;76:51–61.
25. Le Clair EE, Bonfiglio L, Tuan RS. Expression of the paired-box genes Pax-1 and Pax-9 in limb skeleton development. Dev Dyn 1999;214:101–115.
26. Dahl E, Koseki H, Balling R. *pax* genes and organogenesis. BioEssays 1997; 19:755–765.
27. ten Berge D, Brouwer A, Korving J, Martin JF, Meijlink F. *Prx1* and *Prx2* in skeletogenesis: roles in the craniofacial region, inner ear and limbs. Development 1998;25:3831–3842.
28. Hirsch M-R, Valarché I, Deagostini-Bazin H, Pemelle C, Joliot A, Gordis C. An upstream regulatory element of the NCAM promoter contains a binding site for homeodomain. FEBS Lett 1991;287:197–202.
29. Prickett PM. *Hox* and vertebrate skeletogenesis: the long and the short of it. BioEssays 1995;17:750–753.
30. Martin JF, Bradley A, Olson EN. The *paired*-like homeo gene *MHox* is required for early events of skeletogenesis in multiple lineages. Genes Dev 1995;9: 1237–1249.
31. ten Berge D, Brouwer A, Rahi SF, Guénet J-L, Robert B, Meijlink F. Mouse *Alx3*: an *aristaless*-like homeobox gene expressed during embryogenesis in ectomesenchyme and lateral plate mesoderm. Dev Biol 1998;199:11–25.
32. Hudson R, Taniguchi-Sidle A, Boras K, Wiggan ON, Hanel PA. Alx-4, a transcriptional activator whose expression is restricted to sites of epithelial-mesenchymal interactions. Dev Dyn 1998;213:158–169.
33. Launay C, Fromentoux V, Thory C, Shi DL, Boucaut JC. Comparative analysis of the tissue distribution of 3 fibroblast growth-factor receptor messenger-RNAs during amphibian morphogenesis. Differentiation 1994;58:101–111.
34. Chimel-Monroy J, Diaz de León L. Expression of N-cadherin, N-CAM, fibronectin and tenascin is stimulated by TGF-β1, β2, β3 and β5 during the formation of precartilage condensations. Int J Dev Biol 1999;43:59–67.
35. Tavella S, Raffo P, Tacchetti C, Cancedda R, Castagnola P. N-CAM and N-cadherin expression during in vitro chondrogenesis. Exp Cell Res 1994; 215:354–362.
36. Koyama E, Leatherman JL, Shimazu A, Hyun Duck N, Pacifici M. Syndecan-3, tenascin-C, and the development of cartilaginous skeletal elements and joints in chick limbs. Dev Dyn 1995;103:152–162.
37. Gould SE, Upholt WB, Kosher RA. Characterization of chicken syndecan-3 as a heparan sulfate proteoglycan and its expression during embryogenesis. Dev Biol 1995;168:438–461.
38. Perantoni AO, Dove LF, Karavanova I. Basic fibroblast growth factor can mediate the early inductive events in renal development. Proc Natl Acad Sci USA 1995;92:4696–4700.
39. McGonnell IM, Clarke JDW, Tickle C. Fate maps of the developing chick face: analysis of expansion of facial primordia and establishment of the primary palate. Dev Dyn 1998;212:102–118.
40. Kim H-J, Rice DPC, Kettunen PJ, Thesleff I. Fgf-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. Development 1998;125:1241–1251.
41. Koyama E, Shimazu A, Leatherman JL, Golden EB, Nah HD, Pacifici M. Expression of syndecan-3 and tenascin-C: possible involvement in periosteum development. J Orthop Res 1996;14:403–412.
42. Gluhak J, Mais A, Mina M. Tenascin-C is associated with early stages of chondrogenesis by chick mandibular ectomesenchymal cells in vivo and in vitro. Dev Dyn 1996;205:24–40.
43. Saga Y, Yagi T, Ikawa Y, Sakakura T, Aizawa S. Mice develop normally without tenascin. Genes Dev 1991;5:1821–1831.
44. Buchberger A, Schwarzer M, Brand T, Pabst O, Seidl K, Arnold H-H. Chicken winged-Helix transcription factor cFKH-1 prefigures axial and appendicular skeletal structures during skeletal embryogenesis. Dev Dyn 1998;212: 94–101.

45. Labbé E., Silvestri C, Hoodless PA, Wrana JL, Attisano L. Smad2 and Smad3 positively and negatively regulate TGF-β-dependent transcription through the forkhead DNA-binding protein FAST2. Mol Cell 1998;2:109–120.
46. Ida K, Koseki H, Kakinuma H, Kato N, Mizutani-Koseki Y, Ohuchi H, Yoshioka H, Noji S, Kawamura K, Kataoka Y, Ueno F, Taniguchi M, Yoshida N, Sugiyama T, Miura N. Essential roles of the winged helix transcription factor MFH-1 in somite formation and skeletogenesis. Development 1997;124:4627–4638.
47. Ng L-J, Wheatley S, Muscat GEO, Conway-Campbell J, Bowles J, Wright E, Bell DM, Tam PPL, Cheah KSE, Koopman P. SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. Dev Biol 1997;83:108–121.
48. Csaljesi P, Brown D, Ligon KL, Lyons GE, Copeland NG, Gilbert DJ, Jenkins NA, Olson EN. Sclerazis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. Development 1995;121:1099–1110.
49. Kanzler B, Kuschert SJ, Liu Y-H, Mallo M. *Hoxa-2* restricts the chondrogenic domain and inhibits bone formation during development of the branchial arches. Development 1998;25:2587–2597.
50. Yokouchi Y, Nakazato S, Yamamoto M, Goto Y, Kameda T, Iba H, Kuroiwa A. Misexpression of Hoxa-13 induces cartilage homeotic transformation and changes in cell adhesiveness in chick limb buds. Genes Dev 1995;9:2509–2522.
51. Newman SA: Sticky fingers—Hox genes and cell adhesion in vertebrate limb development. BioEssays 1996;18:171–174.
52. Jung J-C, Tsonis PA. Role of 5′ *HoxD* genes in chondrogenesis in vitro. Int J Dev Biol 1998;42:609–615.
53. Koyama E, Leatherland JL, Noji S, Pacifici M. Early chick limb cartilaginous elements possess polarizing activity and express *Hedgehog*-related morphogenetic factors. Dev Dyn 1996;207:344–354.
54. Goff DJ, Tabin CJ. Analysis of *Hoxd-13* and *Hoxd-11* misexpression in chick limb bud reveals that *Hox* genes affect both bone condensation and growth. Development 1997;124:627–636.
55. Davis AP, Witte DP, Hsieh-Li HM, Potter SS, Capecchi MR. Absence of radius and ulna in mice lacking *hoxa-11* and *Hoxd-11*. Nature 1995;375: 791–795.
56. Favier B, Rijli FM, Fromental-Ramain L, Fraulob V, Chambon P, Dollé P. Functional cooperation between the non-paralogous genes *Hoxa-10* and *Hoxd-11* in the developing fore-limb and axial skeleton. Development 1996; 122:449–460.
57. Blanco MJ, Misof BY, Wagner GP. Heterochronic differences of *Hoxa-11* expression in *Xenopus* fore-limb and hind-limb development—Evidence for lower limb patterning of the Anuran ankle bones. Dev Genes Evol 1998;208: 175–187.
58. Langille RM. Chondrogenic differentiation in cultures of embryonic rat mesenchyme. Microscopy Res Tech 1994;28:455–469.
59. Duprez D, Bell EJdeH, Richardson MK, Archer CW, Wolpert L, Brickell PM, Francis-West PH. Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. Mech Dev 1996;57: 145–157.
60. Urist MR, Maeda H, Sharnie AN, Teplica D. Endogenous bone morphogenetic protein expression in transplants of urinary bladder. Plast Reconstr Surg 1998;101:408–415.
61. Urist MR, Raskin K, Goltz D, Mariokel K. Endogenous bone morphogenetic protein—immunohistochemical localization in repair of a punch hole in the rabbit's ear. Plast Reconstr Surg 1997;99:1382–1389.
62. Tintut Y, Parhami F, Bostrom K, Jackson SM, Demer LL. cAMP stimulates osteoblast-like differentiation of calcifying vascular cells—Potential signalling pathway for vascular calcification. J Biol Chem 1998;273:7547–7553.
63. George EL, Georges-Labouesse EN, Patel-King RS, Rayburn H, Hynes RO. Defects in mesoderm, neural tube and vascular development in mouse embryo lacking fibronectin. Development 1993;119:1079–1091.
64. Gehris AL, Stringa E, Spina J, Desmond ME, Tuan RS, Bennett VD. The region encoded by the alternatively spliced exon IIIa in mesenchymal fibronectin appears essential for chondrogenesis at the level of cellular condensation. Dev Biol 1997;190:191–205.
65. Downie SA, Newman SA. Different roles for fibronectin in the generation of fore and hind limb precartilage condensations. Dev Biol 1995;172:519–530.
66. Goldstein RS, Avivi O, Geffen R. Initial axial-level-dependent differences in size of avian dorsal root ganglia are imposed by the sclerotome. Dev Biol 1995;168:214–222.
67. Richman J, Mitchell PJ. Craniofacial development: Knockout mice take one on the chin. Curr Biol 1998;384–387.
68. Gendron-Maguire M, Mallo M, Zhang M, Gridley, T. *Hoxa-2* mutant mice exhibit homeotic transformation of skeletal elements derived from cranial neural crest. Cell 1994;75:1317–1331.
69. Rijli FM, Mark M, Lakkaraju S, Dierich A, Dollé P, Chambon P. A homeotic transformation is generated in the rostral branchial region of the head by disruption of *Hoxa-2*, which acts as a selector gene. Cell 1994;75:1333–1349.
70. Smith KK, Schneider RA. Have gene knockouts caused evolutionary reversals in the mammalian first arch? BioEssays 1998;20:245–255.
71. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 1996;73:613–622.
72. Chung U-I, Lanske B, Lee K, Li E, Kronenberg H. The parathyroid hormone/parathyroid hormone-related peptide receptor coordinates endochondral bone development by directly controlling chondrocyte differentiation. Proc Natl Acad Sci USA 1998;95:13030–13035.
73. Atkinson BL, Fanto KS, Benedict JJ, Huffer WE, Gutierrez-Hartmann A Combination of osteoinductive bone proteins differentiates mesenchymal C3H/10T 1/2 cells specifically to the cartilage lineage. J Cell Biochem 1997;65; 325–339.
74. Shukunami C, Ohta Y, Sakuda M, Hiraki Y. Sequential progression of the differentiation program by bone morphogenetic protein 2 in chondrogenic cell line Atdc 5. Exp Cell Res 1998;41:1–11.
75. Brunet LJ, McMahon JA, McMahon AP, Harland RM. Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. Science 1998; 280:1455–1457.
76. Pizette S, Niswander L. BMPs negatively regulate structure and function of the limb apical ectodermal ridge. Development 1999;126:883–894.
77. Roark EF, Greer K. Transforming growth factor-β and bone morphogenetic protein-2 act by distinct mechanisms to promote chick limb cartilage differentiation in vitro. Dev Dyn 1994;200:103–116.
78. Merino R, Ganan Y, Macias D, Economides AN, Sampath KT, Hurle JM. Morphogenesis of digits in the avian limb is controlled by FGFs, TGFβs, and noggin through BMP signalling. Dev Biol 1998;200:35–45.
79. Fang J, Hall BK. Differential expression of neural cell adhesion molecule (NCAM) during osteogenesis and secondary chondrogenesis in embryonic chick. Int J Dev Biol 1995;9:519–528.
80. Feng J, Hall BK. Chondrogenic cell differentiation from membrane bone periostea. Anat Embryol 1997;96:349–362.
81. Cremer H, Lange R, Christoph A, et al. Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature 1994;367:455–459.
82. Ducy P, Zhang H, Geoffroy V, Ridall AL, Karsenty G. Osf2/cbfa1: a transcriptional activator of osteoblast differentiation. Cell 1997;89:747–754.
83. Ducy P, Karsenty G. Genetic control of cell differentiation in the skeleton. Curr Opin Cell Biol 1998;10:614–619.
84. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao Y-H, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of *cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997;89:755–764.